ROLE OF METAL IONS IN FERMENTATIVE METABOLISM OF YEAST

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed....[redacted] Date....November, 1996

Director of Studies.

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ABSTRACT

This thesis concerns the influence of several metal cations on the growth and fermentative metabolism of industrial strains of yeast. Experiments were conducted in laboratory growth media (both synthetic and complex) and industrial fermentation media (molasses and malt wort) in order to investigate influences of K^+, Mg^{2+}, Ca^{2+} and Zn^{2+} on ethanol production by several strains of the yeast species, *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* and *Schizosaccharomyces pombe*. Mg^{2+} supplementation studies in the media revealed positive effects on cell growth and ethanol production of all yeasts studied. Although differences existed between yeast species with regard to fermentation, the yeast *Saccharomyces cerevisiae*, distillers strain DCL'M', was found to be the highest ethanol producer in all media studied.

Defined media experiments were designed which mimicked high, intermediate and low K^+, Ca^{2+} and Mg^{2+} levels reported in sugarcane molasses and Ca^{2+}, Mg^{2+} and Zn^{2+} in malt wort in order to investigate their interactive effects on alcoholic fermentation. Subsequent analysis of fermentations revealed that the yeast (distillers strain of *S.cerevisiae*) produced higher alcohol levels in the presence of higher levels of K^+ and Mg^{2+} in synthetic molasses and Mg^{2+} and Zn^{2+} in synthetic malt wort. Analysis of variance showed that all two-factor and three-factor interactions were significant but the main effect of Ca^{2+} was not significant at higher levels of Mg^{2+} in synthetic molasses wort. Although there were significant interactions in synthetic malt wort between Zn^{2+}* Ca^{2+} (* = interaction) and Ca^{2+}*Mg^{2+}*Zn^{2+}, neither Ca^{2+} nor Mg^{2+}*Zn^{2+} exhibited significant interactions. The estimated quadratic response surface for ethanol production in molasses in the presence of low, intermediate and high levels of magnesium (16, 70 and 270 ppm, respectively) fitted well (r^2 = 81.9, 83.3 and 83.5, respectively), and the overall regression model was statistically significant (each $p<0.01$). Maximum predicted ethanol yields were found from the response surface at respective combinations of $K^+/Mg^{2+}/Ca^{2+}$, at 2159/270/1118 ppm, 5102/70/509 ppm and 7231/16/1006ppm. The predicted values were 7.75, 7.49 and 5.50 (%v/v), respectively and confirmatory

experimental yields (7.88, 7.08 and 5.63 (%v/v), respectively) were within their 95% prediction interval. Similarly for malt wort fermentations, models showed high coefficients of determination (r^2 = 86.3, 81.9 and 81.9) under high, intermediate and low levels of Zn^{2+}, respectively. It is therefore suggested that such statistical modelling could prove a useful tool in predicting ethanol yields from fermentation media with known levels of K^+, Mg^{2+} and Ca^{2+} and Zn^{2+}. The studies on the influence of Mg^{2+} on ethanol tolerance in yeast revealed that Mg^{2+} supplemented yeast (*Saccharomyces cerevisiae* and *Kluvyromyces marxianus*) maintained higher cell viabilities, produced more ethanol and withstood higher levels of added ethanol than the unsupplemented yeast.

It is concluded that the experimental findings reported in this thesis, particularly with regard to the beneficial effects of Mg^{2+} on yeast fermentation and ethanol tolerance, are directly pertinent to yeast biotechnologies concerned with the commercial production of ethanol.

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CHAPTER 1: Introduction

1.1. The importance of yeast in biotechnology

Yeasts are unicellular eukaryotic microfungi which naturally live either as saprophytes or parasites, but which possess a wide range of uses in biotechnological industries and have immense potential for future exploitation. Yeast has been exploited by mankind for several thousand years, since the dawn of civilisation. The earliest recorded example is ethanol production known to Babylonians around 6000 B.C. When agricultural practices became established, crops were processed into beverages and foods and it is known that yeasts were an essential part of many of those processes (Davenport, 1981). The name yeast derives from the Greek, Yen -"I boil" or French, levure, Latin, levere - "to raise". The term merely explains what it really does. Until Van Leeuwenhook in 1680 looked at yeast through his invented microscope nobody knew yeast as very small spherical or oval bodies. In 1860 Pasteur concluded that without living yeast, fermentation does not take place (Davenport, 1981). The typical yeast cell is an isolated spiracle to oval or sometimes rather elongated cell, especially in old cultures. The average yeast cell is an ellipsoidal of size 7.2×5.6 μ, area, 118μ^2, volume, 118μ^3 and weight 1.3 $\times 10^{-10}$ g (Davenport, 1981). In the ensuing 8000 years the genus *Saccharomyces* has played a central role in the commercial exploitation of yeast by mankind. These facultative anaerobes utilise the Embden-Meyerof-Parnas (EMP) pathway to convert sugars to pyruvic acid. The pyruvic acid is then reductively decarboxylated to give rise to one molecule each of ethanol and carbon dioxide. This simple way of fermenting glucose to ethanol and carbon dioxide is the foundation for major food industries such as baking and brewing. Modern taxonomists say that there are over 300 genera of yeast and the genus *Saccharomyces* has often been referred to as the oldest plant cultivated by man (Berry *et al*, 1987).

The development of brewing and baking yeast strains with improved fermentation characteristics occured with the advent of modern molecular techniques and yeast biotechnology took a new turn by artificially changing the genetic constitution of yeast. Use of yeast for the commercial production of a vaccine against hepatitis B virus could

be considered as a remarkable achievement of modern yeast biotechnology. Yeasts have also become important model systems for basic research into the biology of eukaryotic cells. In the 1940s, *Saccharomyces cerevisiae* became a subject of genetic research and with the development of molecular genetic tools, changes in fundamental research based on the available knowledge of biochemistry, physiology and genetics occurred. The ability to rationally manipulate all aspects of gene expression by *in vitro* genetic techniques offers *S.cerevisiae* a unique place among eukaryotic model systems. Genetically-engineered yeast products are attractive since yeast generally has no pathogenic relationship with Man (with a few notable exceptions such as *Candida albicans*) and prokaryotes such as *E.coli* are not always suitable for all types of genetically engineered products. Today yeast has been widely exploited in modern biotechnological research for the production of a number of health care products like insulin, interferon, serum albumin, antitripsin, lysosyme, streptomycin, IgG , light and heavy chain circumporozite protein, capsid peptide, etc. (Seymour and Welch,1987). In the brewing industry, biotechnological methods have been used to introduce genes for: flocculance (Stewart, 1981), mellibiose or dextrin utilisation (Panchal *et al* 1984), ethanol tolerance (Seki *et al,* 1983), application of maltose utilisation rate by increased gene dosage (Mowshowitz, 1979) and temperature tolerance. Application of recombinant technology to yeast has led to the development of a number of new products (Barr *et al,* 1989) as outlined below (Table 1.1).

Table 1.1 New products developed from yeast using recombinant technology

Product area	Product	Uses
Food	Rennin	Cheese production
Food	Improved Brewer's yeast	Amylolytic activities
Pharmaceutical	Hepatitis-B virus surface antigen	Hepatitis vaccine
Pharmaceutical	Circumporozoite protein from *Plasmodium vivax*	Malaria vaccine
Pharmaceutical	HIV envelope genes	AIDS vaccine

1.2. Yeast fermentative metabolism and its regulation

Yeast, like other living organisms, must obtain all the substances required for its survival and reproduction from the environment. Therefore, media which are used in the laboratory or in industrial production should include all these essential nutrients in order to obtain successful results (Berry and Brown, 1987). These nutrients include carbon, nitrogen, phosphorous, sulphur, potassium, magnesium, calcium, chlorine, sodium, barium, zinc, iron, manganese, copper, cobalt, nickel, arsenic, lead, iodine, molybdate, boron, aluminium, chromium, vanadium and a number of vitamins including: inositol, pantothenate, pyridoxine, thiamine, nicotinic acid and biotin. (Becker *et al* 1971; Henry, 1983; Kamihara and Nakamura, 1982; Kulaev and Vagabov, 1983; Maiorella *et al*, 1984; Maw, 1963; Rogers and LiChstein, 1969; Stryer, 1981; Suomalinen and Oura, 1971). Even though yeast has fairly simple growth requirements and can easily be grown in defined media the pattern of yeast growth is highly sensitive to the type and the concentration of the carbon source and the availability of oxygen. It is known that anaerobic fermentation by yeast largely involves the conversion of sugars or similar substrates to ethanol and carbon dioxide through a series of well known enzyme reactions. However, Pasteur (1872, cited by Harrison and Graham, 1967) observed that only about 95% of the glucose utilised was converted to ethanol (48.4%) and CO_2 (46.6%) with 3.3% forming glycerol and 0.6% succinic acid, yeast biomass and a mixture of a higher alcohols. *S.cerevisiae* can also produce the necessary enzymes to breakdown simple sugars like glucose and fructose to CO_2 and H_2O by the process of aerobic respiration. With good aeration glucose is metabolised to pyruvate through the Emden-Meyerhof- Parnas (EMP) pathway and then pyruvate is converted to CO_2 and H_2O by the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (Frankel, 1982). During this metabolic pathway, generation of ATP, formation of reducing power as NADPH and the formation of intermediates necessary for biosynthesis of cellular material takes place. Most of the ATP generation occurs during the oxidation of acetate to CO_2 and H_2O via the TCA cycle and involves oxidative phosphorylation during

transfer of electrons from reduced NADH to O_2. In anaerobic conditions or high glucose medium (due to repression of mitochondrial function) the generation of ATP by respiration cannot occur. Under these conditions the NADH generated in the glyceraldehyde 3-phosphate to diphosphoglycerate step can only be oxidised by passing on the H^+ atoms to acetaldehyde resulting in the generation of ethanol. The influence of oxygen and glucose in the regulation of yeast metabolism is well known. The reduction in the rate of glycolysis when yeast is grown in the presence of oxygen was reported by Pasteur and has been referred to as the Pasteur effect. The repression of mitochondrial function when yeast is grown in high levels of glucose has been referred to as the Crabtree effect (or the glucose effect or catabolic repression) (Fiechter *et al,* 1981). In the catabolism of glucose to pyruvate via glycolysis, Mg^{2+} is absolutely required for the activities of the key enzymes: hexokinase, phosphofructokinase, phosphoglycerate kinase, enolase, and pyruvate kinase. Subsequent metabolism of pyruvate, either aerobically via pyruvate dehydrogenase multienzyme complex or anaerobically via pyruvate decarboxylase, also requres Mg^{2+} (Walker, 1994). There appear to be two mechanisms by which Mg^{2+} can activate enzymes. Firstly, Mg^{2+} is involved in the formation of an active substrate and secondly in the formation of an active enzyme. An example of the first mechanism is the binding of Mg^{2+} to ATP to yield the $Mg(ATP)^{2-}$ complex. This binding renders the terminal phosphoryl group more succeptible to a nucleophilic attack from a co-substrate due to the electrophilic nature of the Mg. The $Mg(ATP)^{2-}$ complex appears to be the substrate for creatine kinase and it also activates isocitrate, the substrate of isocitrate dehydrogenase (Heaton, 1990).

1.2.1. Industrial significance of fermentative metabolism by yeast

The making of alcohol by distillation of fermented carbohydrate containing materials must be one of the earliest cases of the preparation of a chemical compound in almost the pure state (Harrison and Graham, 1967). The economic importance of ethanol is considerable. The annual world output of potable sprit being equivalent to about 1.5 million tons of pure ethanol and industrial alcohol several million tons, of which about

1.3 million tons were made by fermentation processes in 1963 (World wide survey of fermentation industries, 1966, cited by Harrison and Graham, 1967). In 1984, the total ethanol production in the U.S. was 300 million gallons. In 1989, West Germany produced 6 million gallons of ethanol for fuel purposes (Greenberg, 1981; Heaford, 1989). In technologically advanced countries ethanol is produced by synthetic processes using petrochemicals as the base material, while developing countries, including Sri Lanka, rely solely upon fermentation alcohol. Ethanol has been used as an alternate fuel to petrol in internal combustion engines for many years since the fossil oil prices began to rise. Several countries have already implimented an ethanol programme as a viable alternative to petroleum oil. For example, apart from USA and Europe, in Brazil the national alcohol commission was established in 1975 and decreed that petrol was to be supplemented and eventually replaced by biofuels. Other countries such as Australia, Thailand, South Africa, Papua New Guinea, and the Philippines have embarked on feasibility studies (Jones *et al*, 1981). Therefore, the current world ethanol production should be much higher than the above recorded levels.

Substrates for fermentation are generally one or more of the carbohydrates; sucrose, starch and cellulose (after hydrolysis) or lactose which are widely available as by-products of agricultural industries in most countries. For example, sucrose containing cane or beet molasses, cannery wastes, whey (a refuse of the cheese industry), starch hydrlysates (based on maize, sorghum, cassava, potatoes or other tuber crops), and cellulose-containing wood wastes (bagasse, straw, and municipal waste). Sugars in these substrates or derevated from them following hydrolysis are initially metabolised by yeast using the glycolytic pathway and finally converted to ethanol.

The fermentation of sucrose to ethanol by the yeast *Saccharomyces cerevisiae* can be described by the following equation:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4\,C_2H_5OH + 4\,CO_2$$

342 g 18 g 184 g 176 g

This equation shows that 54% of the sucrose utilised by the yeast can theoretically be converted to ethanol. However, not all of this 54% will produce ethanol as some of the sucrose will be used in the production of cells as it serves as the carbon source for growth of the yeast. Further, some of the sucrose is also used in the formation of by-products such as acetaldehyde, glycerol, succinic acid and fusel oil. In practice, about 93-95% of the theoretical ethanol is obtained. It is vital to keep the conversion efficiency at a high rate as it reduces the residual sugar concentration in the fermented broth. Higher ethanol concentration in the medium leads to reduction in distillation costs which are approximately 40% of the cost of ethanol production. (Smith,1979). Industrialists use a number of methods to achieve this objective including: use of fermentatively efficient yeast strains, ethanol and osmotolerant superior yeast strains and optimization of fermentation parameters, including temperature, pH, dissolved oxygen and nutrients.

1.3. Role of cations in yeast physiology

1.3.1 General requirements for metals in yeast physiology

Since the yeast cell contains various metal nutrients as constituents these play a major role in growth and metabolism. Table 1.2 shows the average elemental composition of *Saccharomyces* species (Jones and Greenfield, 1984).

Table 1.2 Average elemental composition of Saccharomyces species

Element	Average value[g(100g)$^{-1}$ dry weight]
Potassium	2.2
Phosphorus	1.6
Sulphur	300.10^{-3}
Magnesium	270.10^{-3}
Sodium	60.10^{-3}
Calcium	50.10^{-3}
Barium	15.10^{-3}
Zinc	12.10^{-3}
Iron	10.10^{-3}
Copper	5.10^{-3}
Manganese	3.10^{-3}
Cobalt	$0.5.10^{-3}$
Nickel	250.10^{-6}
Arsenic	180.10^{-6}
Lead	150.10^{-6}
Iodine	$125.10.^{-6}$
Molybdate	7.10^{-6}
Boron	5.10^{-6}
Aluminium	1.10^{-6}
Chromium	10.10^{-26}
Vanadium	5.10^{-26}

The concentration of some of the bulk cations and requirements of yeast are shown in Table 1.3 (Eyben *et al*, 1988).

Table 1.3 Concentration and requirements of bulk cations of yeast

Cation	Concentration mg/l	Requirement mg/l
K	224-550	78-56
Na	17-30	-
Ca	23-35	10-20
Mg	67-100	48-97
Zn	0.09-0.15	0.005-0.523 (strain dependent)

A number of scientists who have been working on the effects of inorganic ions on the growth and fermentation of yeast have shown the importance of these cations in fermentation media (Dombek and Ingram, 1986; Walker *et al*, 1990).

Inorganic cations exert their influence not only on carbohydrate assimilation and metabolism but also on the ability of yeast to tolerate ethanol. Most of the essential cations influence enzyme activity by acting as common catalytic centres. While Zn^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} act as a common enzyme catalyst, Mg^{2+} is considered as the most common enzyme activator. Most of the cations act as structural components in that they help to utilise negative electrostatic forces in various cell components. K^{+} and Mg^{2+} are found to act on such forces in polyphosphate, DNA, RNA and proteins. Ca^{2+}, Mg^{2+}, and Zn^{2+} are found in combination with membranes as membrane phospholipids (Lewis *et al,* 1978; cited by Jones and Greenfield, 1984).

Among the bulk cations of yeast potassium plays a central role in the regulation of yeast growth and fermentation under both aerobic and fermentative conditions. It also plays a major role in divalent cation uptake with excretion $2K^{+}$ for each divalent cation taken up. Its presence reduces the cellular concentration of inhibitory ions. Low pH favours the higher optimum levels of this cation in yeast cells. During fermentation, yeast cells take up more $H_2PO_4^{-}$ and K^{+} is essential for the uptake of these ions during fermentation. The yeast membrane is asymmetric with respect to K^{+}, choosing it at the outer surface in preference to Na^{+} and discriminating against it at the inner surface where Na^{+} or H^{+} is

preferentially chosen. During the K^+ uptake process, membrane H^+ ATP-ase provides the necessary energy and Na^+/K^+ are effluxed from the cells. Uptake of K^+ can be inhibited by other alkali metal ions. Since K^+ stimulates glycolysis its presence elevates the levels of NAD(P)H, ATP, ADP and Pi in yeast cells (Suomalainen and Oura, 1971; Rodriguez-Navarro and Asensio, 1977).

Deficiency of K^+ in fermentation media reduces the biomass yield as well as the fermentation rate. The optimum growth of *S.cerevisiae* was recorded at 2mM-4mM concentrations and deficiencies caused decreased growth and fermentation rates (Jones and Greenfield, 1984). The K^+ requirement increases with the decrease of Na^+ concentration in the medium. Total inhibition of growth and fermentation occurs above 60mM K^+ . In brewing, wort K^+ stimulates utilisation of maltose and maltotriose. There is a correlation between specific ethanol productivity and K^+ under aerobic conditions. Increased K^+ levels increase glucose assimilation and ethanol production (Wumpelmann and Kjaergarrd, 1979).

Magnesium is another bulk cation in yeast and one of the most abundant cations on the earth surface, sea water, human body and in living cells (Walker, 1994). It serves as an intracellular cation in a number of physiological activities including: a cofactor for over 300 enzymes, conformation of nucleic acids and maintenance of the structural integrity of cell membrane (Walker, 1994). Magnesium is at the centre of fermentative metabolism as it participates in a number of enzymatic activities viz; stimulation of the synthesis of essential fatty acids, alleviation of the inhibitory effects of alkali metals, activation of glycolysis by stimulation of pyruvate carboxylase, phosphate transferase and decarboxylase activity, regulation of the cellular ionic levels, stimulation of $H_2PO_4^-$ uptake, activation of membrane ATPase, optimisation of membrane protein-protein functions and lipid-protein interactions to maintain the integrity and the permeability of membrane and regulation of divalent cation transport. Walker and Duffus (1980) and Walker *et al* (1990) reported the influence of Mg^{2+} on the regulation of the cell cycle of *Schizosaccharomyces pombe* and possibility of exploiting this knowledge to maximise

production processes which involve fed-batch systems where the culture growth rate is important. Further, Mg^{2+} also has potential to optimize the production of ethanol as it may be a cell cycle-linked secretory process. According to Walker *et al* (1982; 1990), Mg^{2+} plays a central role in carbohydrate metabolism and therefore ethanol production in *S.pombe* by dictating the predominant route of glucose catabolism. Further, they showed that by limiting the availability of magnesium to yeast cells grown under respirofermentative conditions, cells switched from being predominantly fermentative to predominantly respiratory. Based on those studies they have developed a hypothesis linking Mg^{2+} availability to regulation of respirofermentative metabolism as shown in Fig 1.1.

Fig 1.1 Hypothesis linking Mg availability to regulation of respirofermentative metabolism in yeast (Walker *et al*, 1990)

GLUCOSE

(Aerobic conditions)

glycolysis

⇓

pyruvate decarboxylase ⇐ **PYRUVATE** ⇒ pyruvate dehydrogenase

(PDC) (PDH)

⇓ ⇓

⇓ **HYPOTHESIS** ⇓

fermentative route **PDC & PDH are rate** respiratory route

⇓ **limiting enzymes in** ⇓

⇓ **yeast sugar catabolism** ⇓

⇓ **and are regulated by Mg** ⇓

availability

ETHANOL H_2O

CO_2 CO_2

2 ATP 36 ATP

The hypothesis proposes that the key enzymes of pyruvate metabolism, namely pyruvate decarboxylase (PDC) and pyruvate dehydrogenase (PDH) , possess different affinities for intracellular free Mg^{2+} ions. Thus, if PDC exhibited a high K_m *in vivo* for Mg^{2+}, fermentation would not predominate below certain threshold levels of free Mg^{2+}, whereas if PDC exhibited a low K_m for Mg^{2+}, respiration would predominate even under Mg-limited growth conditions (Walker *et al*, 1990).

It was reported that there is 10m moles Mg/100g dry wt. present in the yeast plasma membrane which is 20 times more than Ca^{2+} (Lewis, Somer and Patel, 1978; Diamond and Rose, 1970; Lotan *et al*, 1976). Magnesium activity appears to influence attenuation rates and the final yields of anaerobic cultures of *S.cerevisiae* (Walker *et al,* 1990, Dombek and Ingram, 1986). Elevated Mg^{2+} levels have been shown to increase the rate of ethanol production leading to higher final ethanol concentrations. Therefore, Mg^{2+} influences key enzymes which govern sugar metabolism.

Calcium is also another bulk cation in yeast but according to Jones and Greenfield (1984) it is not essential for the growth of *S.cerevisiae*, although Ca^{2+} ions have a slight growth stimulatory effect. Ca^{2+} is taken up only by actively growing cells and is incorporated into newly synthesised structural materials of the yeast cells. Ca^{2+} appears to be involved in the linkage of binding membrane phospholipid to proteins and it has a high affinity of binding to membrane proteins compared to Mg^{2+} (Lewis *et al,* 1978). The K_T value for the uptake of Ca^{2+} by *S.cerevisiae* was reported to be higher than 500μM. Membrane-bound Ca^{2+} appears central in the regulation of lipid-protein interactions. The major effect of the addition of Ca^{2+} is an increase in biomass and in protecting membrane structure and permeability against adverse environmental conditions. *S.cerevisiae* has a very low growth requirement for Ca^{2+} and concentrations above 1mM cause inhibition of amino acid uptake whilst concentrations above 25mM cause reduction in cell growth and ethanol production. According to Saltukoglu and Slaughter (1983) Ca^{2+} competes with Mg^{2+} and prevents yeast growth almost entirely when present at a ten fold excess. At a two to three times excess the effect was more on

length of the lag phase and mean generation time than the total amount of growth. As industrial media like sugarcane molasses are rich in Ca^{2+} this matter should be considered carefully before the commencement of any industrial production as it may result in economic losses.

The other bulk cation concerned in yeast fermentative metabolism is zinc and the importance of zinc and other micronutrients has been known for several years and considered to be low in toxicity to fungi and yeast (Somers, 1961). These nutrients function as cofactors of prosthetic groups and coenzymes. Among the micronutrients, zinc plays an essential role in yeast metabolism being a part of over 200 enzyme systems including 4 known to be essential. Zn^{2+} is essential for many cellular functions and second only to iron interms of cellular functions (Thoman, 1983). After the finding of the first zinc metallo enzyme, carbonic anhydrase, by Keilin and Manns (1940) research on metalloenzymes has been accelerated in many disciplines. The role of zinc in metalloenzymes can be divided in to four categories: catalytic, structural, regulatory and non-catalytic. The zinc metalloenzymes in yeast are categorised as oxidoreductases, transferases, hydrolyases, isomerases and ligases. Zinc is a 11B transition element with a completed **d** subshell and two additional **s** electrons which chemically combine in the +2 oxidation state, but is neither oxidised nor reduced in biological reactions (Thoman, 1983). Zn^{2+} is essential for the activity of Zn^{2+} containing enzymes like glyceraldehyde phosphate dehydrogenase, alcohol dehydrogenase, cysteine desulphydrase, aldolase etc. Cells cannot grow or ferment without Zn^{2+} although a number of other ions can substitute the effect of Zn^{2+}. This cation also takes part in activating riboflavin synthesis, acid and alkali phosphatases and in increasing protein content in fermenting yeast. It also has the capacity of stimulating the uptake of maltose and maltotriose. Yeast cells take up Zn^{2+} rapidly and increase fermentation rate. The activity of this cation is mostly on membrane functions but it can also influence membrane proteins. There are also indications that this cation affects the stimulatory effect of membrane-bound Ca^{2+}. In growth media 4-8μM is found to be optimum although this depends on the strain.

Fermentation studies conducted in brewing wort by Denseky *et al* (1966) using ale yeast have shown the stimulatory effect due to Zn^{2+} at the level of 0.1 to 1ppm. Further, the speed of fermentation increases and fermentation time reduces by approximately 15-20%. and the yeast crops (i.e. yeast separating at the surface) were greater, occurred earlier and the yeast flocculated more quickly. However, the total yeast biomass production was not affected by the availability of Zn^{2+} and no change in flocculation power was detected (Denseky *et al*, 1966).

1.3.2. Uptake and transport of mineral cations by yeast

According to Rose (1976) there are basically five types of solute transport processes that effect movement of solute molecules across biological membranes viz. free diffusion (solutes passes through lipid domains without membrane protein involvement), protein mediated transport, which include; facilitated/non concentrative diffusion process (solutes move down a concentration gradient), active transport (transport against concentration gradient needs metabolic energy), group translocation (solute molecule is chemically modified) and finally pinocytosis.

Cation uptake by yeast is typically biphasic, involving rapid, metabolism-independent binding to cell surfaces, followed by a slower phase of metabolism dependent intracellular uptake (Rothstein *et al*, 1958). Generally, the divalent cation uptake is accompanied by the efflux of monovalent cation K^+, but H^+ for Ca^{2+} and Mg^{2+} when they are absorbed by the monovalent cation transport syst1em (Borst-Pauwels, 1981).

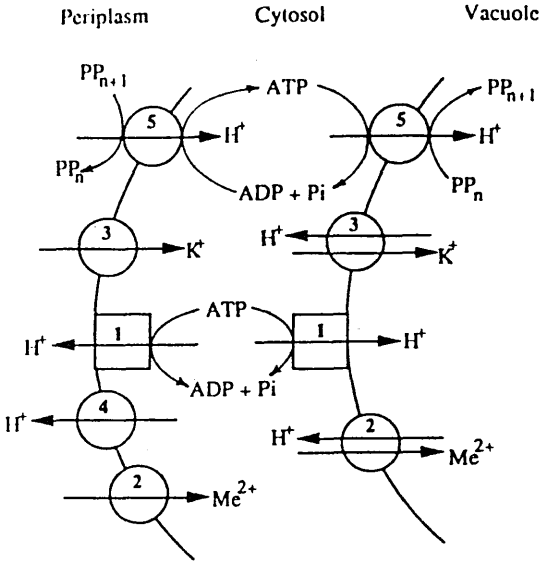
When a particular cation is toxic to yeast cells it tends to maintain a low concentration in the cell. For example Li^+ is toxic to many yeasts and many strains maintain low intracellular concentrations. Kinetic evidence suggested the existence of an active Li^+ efflux system in yeast and the efflux was regulated by the cellular ATP content and intracellular pH (Jones and Gadd, 1990). The uptake of metal cations by *S.cerevisiae* is generally accompanied by potassium efflux which may be an integral part of the

physiological mechanism for maintaining the ionic balance or may be a symptom of ionic balance or membrane disruption and cell death (Passow and Rothstein, 1960). Yeast physiological condition drastically affected the extents of K^+ uptake and excretion (Mochaba *et al*, 1996). Influx of monovalent cations into *S.cerevisiae* cells is mediated by the physiological potassium ion carrier. In this, three distinct sites have been identified by different workers; the first among them called a transport site (Conway and Duggan, 1958), the second a modifier site and the third an activated site (Borst-Pauwels, 1981). All these interact with protons and potassium ions. It is more certain that the three sites comprising the monovalent cation efflux system are real as a simple cubic relationship exits between sodium ion efflux rate and the concentration difference of ions across the plasma membrane (Dee and Conway, 1968). Divalent cation transport in yeast is concentration dependent and protein mediated depending on external concentrations. Although transport may obey Michaelis-Menten kinetics there are extensive variations (White and Gadd, 1987). The reasons for variations in kinetic parameters could be due to: differences between strains and experimental procedures, binding or complexation of cations to cell wall materials, components of the growth medium which may reduce the free ion concentrations, toxic effects of cations and differences in thermodynamic conditions (Norris and Kelly, 1977). There is evidence for the existence of another translocation system for some ions viz. Co^{2+}, Ni^{2+}, Cd^{2+}, Zn^{2+}, Ca^{2+}, Sr^{2+}, as concave Hofstee plots were obtained suggesting operation of two transport systems. Since different workers got two K_T values for Zn^{2+} implying there could be two transport systems for Zn^{2+} (Failla and Weinberg, 1977). Uptake of Mg^{2+}, Mn^{2+}, Sr^{2+} and Zn^{2+} ions is driven by both the proton and potassium gradients across yeast cell membranes whereas that for cobalt and nickel is driven only by the proton gradient (Lichko *et al*, 1980). The proposed mechanism for the uptake of transition metal ions is shown in Fig. 1.2. Microorganisms have selective transport systems for the uptake of the metals of known biological functions with reasonable multiplicity and complexity like K^+ and Fe^{2+}. These systems are highly specific or with broader specificity which can transport cations without specific functions but which stop if accumulated in toxic

concentrations. Clear examples of competition between essential and nonessential metal ions are provided by bacterial transport systems for mono and divalent cations. Thus Ti^{2+} surves as a substitute for the K^{+} transport system in *Streptococus lactis* (Kashket, 1979) and potassium transport systems of *E.coli* (Damper *et al*, 1979). In both cases uptake of Ti^{2+} is inhibited by K^{+}. Therefore Ti^{2+} could be used as a probe for transport of K^{+} in bacteria and yeast. However, in *S.cerevisiae* uptake of divalent cations is active, mediated by at least one transport system specifically for accumulation of divalent cations with a decreasing affinity series Mg^{2+}> Co^{2+}> Zn^{2+}> >Mn^{2+} >Ni^{2+}> Ca^{2+}> Sr^{2+} (Rothstein *et al*, 1958).

Fig 1.2 Model for cellular uptake and trafficking of trasition metal ions

(Okorokov *et al*, 1983, cited by Kosman, 1994; Gadd, 1990).

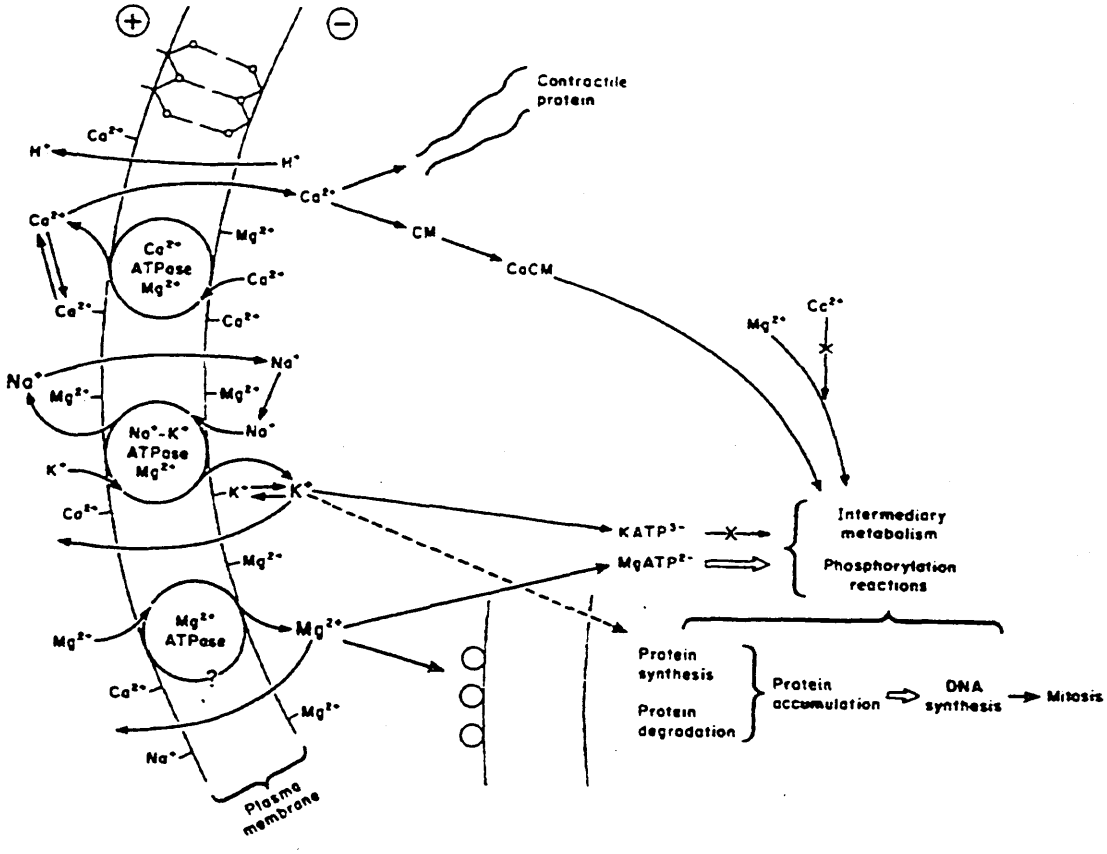


(1) H^+ ATP ase (2) Metal ion transporter coupled directly or indirectly to H^+ and /or K^+ gradient (3)K= transporter or K^+/H^+ antiporter (4) H^+ efflux perhaps coupled to Me^{2+} influx.(5) Polyphosphate kinase acting as a H^+ pump and for retaining metal in the vacuole.

Mg^{2+} and Ca^{2+} can translocate through monovalent carrier of the yeast. Most divalent cations are accumulated into the yeast cell by means of translocation systems that are activated by phosphate uptake (Furhamann and Rothstein, 1968). They also reported that the Zn^{2+} uptake is stimulated by phosphate. However, Pena, (1980) reported that phosphate had no effect on Ca^{2+} uptake. Such experiments reveal that yeast cells selectively accumulate Mg^{2+} and Mn^{2+} to a much greater extent than Ca^{2+} and Sr^{2+}. This difference in accumulation is probably not due to differences in affinity of the divalent cations for the transport mechanism. However, Pena (1975) found a stimulatory effect of K^{+} on phosphate uptake and hypothesised that phosphorylated products were involved in the translocation of divalent cations through the cell membrane. According to Norris and Kelly (1977) the selectivity of yeast cells for divalent cations depends upon the type of yeast and the dissociation constant of the cation. S.*cerevisiae* and *Sporobolomyces roseus* accumulated zinc from zinc containing medium and uptake was biphasic. Further, zinc uptake by viable cells was not accompanied by potassium release in either yeast (Mowll and Gadd, 1983). Studies on *Candida utilis* showed that Zn^{2+} uptake is energy-dependent and is specific for this ion. There is also limited information on the intracellular distribution of Zn^{2+}. According to Failla and Weinberg (1977) the only way *Candida utilis* reduced its intracellular Zn^{2+} was by dilution due to cell growth and division, since no efflux was observed. Zn^{2+} uptake by *S.cerevisiae* has been reported by White and Gadd (1987) who indicated the involvement of a plasma membrane ATPase. They also evaluated the role of K^{+} efflux as a driving force for Zn^{2+} uptake and suggested that the major fraction of K^{+} efflux was unrelated to Zn^{2+} and established that Zn^{2+} does not efflux from *S.cerevisiae*. Uptake of transition metal ions like Zn^{2+} is facilitated in yeast. Early evidence suggested that the yeast *S.cerevisiae* plasma membrane contained a general divalent cation transport system which is selective for Mn^{2+}, Mg^{2+} and active for Co^{2+}, Zn^{2+} and Ni^{2+} . More recent work has demonstrated high affinity and specific uptake systems for Mn^{2+} Cu^{2+} Zn^{2+}, Co^{2+} and Fe^{2+} in yeast (Kosman, 1994).

More recent investigations into the effects of Mg2+ ions, particularly in yeast cells and in fibroblasts, has led to a new concept that identifies a central role for for free Mg2+ in the coordinate control of metabolism, growth and cell proliferation and Fig 1.3 displays the model proposed by Sanui and Rubin (1982) for the role of Mg^{2+} and Ca^{2+} in the regulation of cellular metabolism and proliferation is shown in Fig 1.3.

Fig 1.3 Model for the role of Mg^{2+} and Ca^{2+} in the regulation of cellular metabolism.



At a steady state, the level of intracellular cations is determined by a delicate balance between active and passive processes. An external effector would perturb the plasma membrane, producing a shift in the balance of active and passive transport process and consequently in the levels of intracellular cations. A change in Mg^{2+} results in a modification of the rates of multitude of Mg^{2+} activated reactions. In particular, there would be a change in the amount of Mg^{2+} - ATP complex that is required for rate-

limiting phosphorylation reactions in intermediary metabolism and macromolecular synthesis. The net result would be changes in rates of protein synthesis, DNA synthesis and cell proliferation. Accumulated cation data suggested an inwardly directed Mg^{2+} pump in apparent contradiction to expectations from classic membrane theory (Sanui and Rubin, 1982).

1.3.3. Interaction between cations in yeast physiology

The ability of the microbial cell to deal with the wide range of metal ions required for metabolic functions is an interesting phenomenon. The uptake of metal ions into cells depends on the concentration of the particular ion in its growth environment and the bio-availability which depends on the solubility and properties of ion-complexing ligands. Uptake of a specific metal from the environment is controlled by the cell membrane which can distinguish between specific metal ions and provide a specific transport system for their translocation into intracellular sites. Other metal cations are necessarily excluded from these sites. This precise control of metal binding in biology contrasts with a simple chemical situation (free of forces that affect free movement of ions) where the competition between metal ion for a ligand is normally won by the metal ion which is the strongest Lewis acid of those present. However, cells have the ability to select required cations and insert them into the complexing site (Williams, 1981). During the binding process there is an obvious competition between foreign and native cations and it is possible to arrange suitable conditions for the uptake of foreign cations by the facilitating conditions for them to compete with native ions and finally replace them. Therefore, it is desirable to study such interactions due to a number of reasons. For example, replacement of active ions may result in a perturbation of normal biological function which may lead to useful findings of the functions of the native cation under normal physiological conditions and further it promotes the understading of the toxic effects of metals towards microorganisms. Such studies may also provide valuable medical information. For example, competition between Fe^{3+} and Al^{3+} in neurotoxicity and studies of antitumour properties of Ga^{3+} which arise from the replacement of Fe^{3+} by

Ga^{3+} (Hughes and Poole 1981). Metal ions in biology most frequently bind to donor ligands according to preferences dictated by hard-soft theory of acid and bases. Cations H^+, Na^+, K^+, Mg^{2+}, Mn^{2+}, Al^{3+}, Ga^{3+}, Ca^{2+}, Cr^{2+},Co^{2+}, Fe^{2+} and Ti^{2+} are less easily polarised and small atoms are considered as hard Lewis acids. Large and easily polarisable cations like Cu^{2+} are considered as soft Lewis acids. The cations Zn^{2+}, Cu^{3+}, Fe^{2+} etc. lie in between hard-soft Lewis acids. The ligands that these Lewis acid cations bind to are called as Lewis bases. The Lewis bases for the hard acids are: H_2O, OH^-, $CH_3CO_2^-$, PO_4^{3-} , $ROPO_3^{2-}$, CO_2-, NO^{3-}, ROH, R_2O, NH_3, RNH_2, N_2H_4, RO and Cl^-. For the intermediate Lewis acids the Lewis bases are: NO_2^-, N_2 , SO_3^{2-}, Br^-, N^{2-}, and for soft Lewis acids bases are: R_2S, RS^-, RSH, $(RS0)_2PO_2^-$, SCN^-, H^-, R_3P, CN^-, RNC, $(RO)_2PO)S^-$, CO and R^- (Poole and Gadd, 1989). The binding preference of divalent transition metal cations to a given ligand were first deliniated by Irving and Williams (1953) as follows: $Ca^{2+}<Mg^{2+}<Fe^{2+}<Co^{2+}<Ni^{2+}<Cu^{2+}$and Zn^{2+} has a lower binding preference than Cu^{2+}.

The affinity of a cation for a particular ligand depends on several factors: the selected cation should bind in a similar fashion to the native cation and to achieve that both cations should have the same ionic charges, similar ionic radii and prefer the same cordination numbers, geometrics and ligand types. These ligand preferences will vary with the native metal and the site involved which could be O_2/N_2 (hard) or S (soft) donors. If metals with higher oxidation states such as Fe^{3+} are involved then negatively charged ligands will probably be included in the cordination shell in order to reduce the overall effective charge on the metal centre. The similarity between two cations can be estimated by comparing values of formation constants for their interactions with a range of ligands in solution. During cellular metabolic processes, cations with some similarities may substitute with one another. For example, K^+ which has an ionic radius (i.r) of 1.33 A° could be replaced with Ti^{2+} (i.r 1.40 A°), Mg^{2+} i.r (0.72 A°) could be substituted with Ni^{2+}/Mn^{2+} with 0.60 & 0.80 A° i.r , respectively. Similarly, Ca^{2+} (0.99 i.r) could be substituted with Mn^{2+} or La^{2+} (0.93 & 1.15 i.r) or Cd^{2+} (0.97 i.r).

Further, Zn^{2+} (0.74 i.r) could be replaced with Co^{2+} or Cd^{2+} which have i.r of 0.72 and 0.97 A° , respectively.

Some metals form insoluble complexes with anions present in the media or cells. Therefore, replacement metals can't act independently so that they cause incompatible oxidation-reduction properties and sometime replaced cations may bind to additional sites causing inhibition. The other complication could be that the replacing cation is poorly labile so that exchange of ligands is slow. The rate of entering and leaving a cation from a ligand depends on the polarising power of the cation. Polarising power is the ratio of charge/ionic radius. Generally metallic cations are very labile. The rate of ligand exchange of some cations are: $Al<Fe<Ga<<<Mg^{2+}<<<Zn^{2+}<Ca^{2+}$. The cation Ni^{2+} has similar characteristics to Mg^{2+} but is not a good replacement for Mg^{2+} as Ni^{2+}-substituted Mg^{2+} enzymes give inactive products as Ni^{2+} is not labile enough. However, the enzyme phosphoglucomutase is a rare example for active substitution of Ni^{2+} for Mg^{2+} (Mildvan, 1977). However, Mn^{2+} is a better replacement for Mg^{2+} (Cohn, 1970). When Al^{3+} is present in the medium, ligand exchange rates show an inhibition of Mg^{2+}-dependent enzymes because the very low ligand exchange rates of Al^{3+} and Mg^{2+} can easily be replaced by Al^{3+} in biological systems. Further, Al^{3+} binds more strongly to ATP than does Mg^{2+}. Therefore, very low concentrations of Al^{3+} in the medium can compete with higher concentrations of Mg^{2+} and cause enzyme inhibition (Martin, 1986). Sometimes replacing cations may bind to the ligand in a different manner to the native cation and therefore enzyme activation takes different mechanisms.

Na^{+}, K^{+} and Ca^{2+}, Mg^{2+} in biological systems are classified as bulk elements. In the periodic table these ions fall into s block elements and Na^{+}, K^{+} and Ca^{2+}, Mg^{2+} are categorised as Gp 1A and 11A ions, respectively. In general, most of these metal ions are selectively distributed in cells. Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, and Mo^{2+} are included in 3d transition ions. These cations form stronger complexes than do the 1A and 11A

cations. The above cations, Na^+, K^+, Ca^{2+}, Mg^{2+}, and Al^{3+} prefer oxygen as a ligand while Mn^{2+}, Fe^{2+}, Mo^{2+} etc. prefer $O_2/N_2/S$ and Co^{2+}, Zn^{2+}, Cu^{2+} need N_2/S containing ligands. Because of these preferences biological systems can select essential cations and incorporate into selective sites.

According to Borst-Pauwels (1981), the mutual interactions of divalent cations influence the uptake of divalent cations. The inter-relationship resulting in inhibition of either transport systems is simply a matter of competition between the cations K^+, Na^+ and Ca^{2+}, Mg^{2+} in the medium (Jones and Gadd, 1990). However, for the monovalent cation uptake system Mg^{2+} at 2mM inhibited the uptake of K^+ (5mM) by 30% while Ca^{2+} (2mM) reduced the uptake by 40% with non-competitive effects (Borst-Pauwels, 1981). The competition between Mg^{2+} , Ca^{2+} and K^+ for uptake via monovalent uptake systems is negligible. However, di and polyvalent cations may inhibit monovalent cation uptake by binding to negatively charged groups on the cell membrane and hence decrease the negative charge potential (Borst-Pauwels, 1981). The uptake of Zn^{2+} is increased especially by Cu^{2+} while the uptake of Mn^{2+} is unaffected. The effects of SH groups and amino groups in yeast cells have the capacity to attract Cu^{2+} and Zn^{2+} but not cations like Mn^{2+} which have no affinity for such groups. In *Candida utilis,* Zn^{2+} uptake is very specific and only inhibited by Cd^{2+} but not by Ca^{2+}, Mn^{2+}, Co^{2+} or Cu^{2+}. When there is no added substrate, uptake of Zn^{2+} and Co^{2+} from the medium is influenced by each other but Ca^{2+} inhibits both Zn^{2+} and Co^{2+} uptake (Furhmann and Rothstein, 1968). Mutual interactions between divlent cations may result in inhibition or stimulation of uptake due to indirect effects such as alteration of membrane permeability (Gadd, 1986).

1.3.4. Effects of media composition on ethanol production and tolerance in yeast.

Although no universal definition of ethanol tolerance in yeast exists, generally it could be defined as the ability of a yeast strain to withstand higher levels of ethanol without any deleterious effects to its viability and metabolic activity. There are numerous reports on the effects of ethanol on the specific growth rate , viability and rate of fermentation (Bevan *et al*,1982). Different yeast strains show variations in their ability to withstand ethanol in their environment. While the Sake brewing yeast *S. sak'e* can tolerate more than 20%v/v ethanol in the medium, traditional brewing yeasts usually withstand concentrations of only 4-5%v/v (Casey and Ingledew, 1986). As the ethanol tolerance of yeast has great industrial significance, it is wise to pay attention to both the basic and applied aspects of yeast-ethanol interactions. Casey and Ingledew (1986) have reviewed the effect of ethanol on yeast nutrient uptake mechanisms. Ethanol imbalances the membrane potential and causes liquidization of intracellular phospholipids. Further, many workers have shown that the yeast plasma membrane is the primary target of ethanol. Thomas *et al* (1978), have shown that when yeast cells are enriched with linoleyl or palmitoleyl lipds, they showed more ethanol tolerance. Watson and Cavicchivoli (1983) found a higher ethanol tolerance of yeast grown in fermentation media when enriched with unsaturated fatty acids. As *S. cerevisiae* cannot synthesise unsaturated fatty acids and sterols when they are grown under anaerobic conditions, media should be supplemented with external sources of these compounds. This consideration is highly applicable in industrial situations as large batch fermenters essentially create anaerobic conditions. Some by products: hexanoic, octanoic and decanoic acids which are formed during fermentation also act as inhibitors to yeast growth and fermentation (Price, 1995).

During industrial fermentations, yeast must obtain all the substances required for growth and metabolism from feedstocks such as malt wort, molasses or wine must. In brewing, wort composition greatly influences the fermentation rate, biomass yield, and beer quality. Wort constituents include fermentable carbohydrates, assimilable nitrogen compounds such as amino acids, purine and pyrimidines. The inorganic ions present in

wort are shown in Table 1.4. As amino acids influence growth of yeast the availability of these acids are vitally important. Wort composition is also influenced by wort temperature. Low mash temperatures result in an increase in medium chain-length carbohydrates and therefore higher attenuation and increased levels of flavour compounds (mainly fusel oils) may ensue. Higher wort temperatures may decreases wort carbohydrates, nitrogeneous compounds and increases inhibitory compounds such as caramelised products. The presence of suspended solids also affects yeast growth and the level of fusel oils depress beer head formation while accelerating the yeast autolysis (Harrison and Graham, 1967).

Table 1.4 Metals through the brewing process (Helin and Slaughter, 1977)

Metal	**malt(mg/kg)**	**hops(mg/Kg)**	**wort(mg/l,1040 S.G)**	**beer(mg/l)**
Mg	1018	2205	69.6	60
Ca	544	10270	7.5	10
Al	24.6	192	0.42	0.26
Fe	51.8	361	0.10	0.08
Mn	13.3	82.1	0.15	0.12
Zn	16.4	47.6	0.118	0.036
Cu	4.6	16.9	0.054	0.015
Ni	0.49	2.9	0.02	0.015
Pb	0.20	1.2	0.02	0.02
Co	0.14	<0.05	0.01	<.002

The composition of malt is also affected by the geographic conditions where barley is grown. Studies have shown that there is a very high correlation between Ca^{2+} and Zn^{2+} and the location. Zn^{2+} levels are an important factor since addition of Zn^{2+} into the medium at a rate of 0.2mg/l accelerates fermentation. However, higher Zn^{2+} levels are toxic if the medium is low in Mn^{2+}. Most brewing worts contain suboptimal levels of Zn^{2+}. This could be the reason for the stimulatory effect on fermentation when Zn^{2+} is added in the brewing wort. Binding of Zn^{2+} to various complexing compounds in wort

may reduce its availability to yeast cells (Densky *et al*, 1966).

In brewery fermentations the metal ion composition influences not only the fermentation but also the stability and flavour of the final product. Many malt and wort constitients have a strong metal binding ability and therefore participate in regulating wort ion availibility (Mandl, 1974) According to Stewart *et al,* (1988), media composition has a profound influence upon the ability of a yeast strain to ferment concentrated substrates. Supplementing with peptone-yeast extract, potassium salts or Mg^{2+} has a positve effect upon overall fermentation rate.

Molasses (cane and beet) is a major industrial feedstock in the alcohol industry in most sugar producing countries. Blackstrap molasses is the by product (or end product) of either raw sugar manufacture or refining; it is the heavy viscous liquid separated from the final low-grade massecuite from which no further sugar can be crystallised by the usual methods (Chen and Chen, 1985). As the final molasses is derived from the sugarcane juice it contains most of the nonsugars in juice and a portion of sucrose and other sugars. Therefore, the composition changes with the variety and maturity of cane, climatic and soil conditions, extent of milling and nature of the clarification process. The ash composition is qualitatively like that of the cane juice from which it originated. The ash content in blackstrap varies in the range 10-15%. The composition of ash also varies and analysis has shown that potassium is present in the highest percentage, ranging around 40% (see Table 1.5 and 1.6).

Table 1.5 The constituents of Australian molasses (Chen and Chen, 1985)

Oxide	**Percentage**
SiO_2	1.86-6.60
K_2O	37.48-41.78
CaO	10.27-16.58
MgO	1.53-8.50
P_2O_5	3.69-9.59

Table 1.6. Typical composition (%) of beet and cane molasses (Rosen, 1987)

	Beet	**Cane**
Dry matter	74-78	75
Total sugar	48-52	48-56
Invert sugar	0.2-1.2	15-20
Raffinose	0.5-2	NA
Fermentable sugar	45-47	46-52
Organic non sugars	12-17	9-12
N-containing compounds	6-8	2-3
Betaine	3-4	NA
Glutamic acid	2-3	NA
Organic acids	6-8	3
Gums(etc)	NA	4
Ash	10-12	10-15
Na(% of ash)	0.3-0.7	0.1-0.4
K	2-7	1.5-5
Ca	0.1-0.5	0.4-0.8
Cl	0.5-1.5	0.7-3.0
P	.02-.07	.6-2.0
pH	7-9	5-6
Vitamins(ppm)		
Biotin	0.04-0.13	1.2-3.2
Inositol	6000-8000	6000
Pantothenic acid	58-100	54-64
Thiamin	1.3	1.8
Nicotinic acid	20-45	30-80.

Molasses is used in a number of industrial fermentations since it is rich in sugars and other major micro-nutrients as well as growth factors. However, a number of components such as excess levels of inorganic ions, phenol and indole compounds , carmelisation products, chlorophenols, imidosulphonate products also exert inhibitory effects on fermentation process (Chen and Chen, 1985). The presence of inorganic nutrients mainly N, P, K, Ca, and Mg is essential for the proper growth and fermentation activity of yeast (Harrison and Graham, 1967). Molasses is rich in some of these nutrients and but others should be incorporated into the wort prior to fermentation. Molasses contains Ca^{2+} and Mg^{2+} at variable and suboptimal levels for yeast metabolism and Wolniewicz *et al* (1988) found a positive influence of Mg^{2+} supplementation during molasses fermentation. Nutrient limitation during fermentation is a major factor in declining cell growth rates and ethanol yields, especially in high gravity fermentations (Casey and Ingledew, 1986). Dombek and Ingram (1986) demonstrated the positive influence of Mg^{2+} on yeast fermentation kinetics in both laboratory and industrial media. Although the typical composition of industrial media like molasses shows that they are rich in inorganic nutrients the composition is highly dependent on the variety, location , climate and mainly to the cropping practices. When crops are grown on the same field over and over for generations naturally soils become deficient in most of these nutrients causing defiencies to plants which essentially results in feed stocks becoming nutrient deficient.

When we review the importance of the above major cationic nutrients for yeast fermentations, potassium is abundant in most industrial media and there are very few reports on the impaired fermentations caused by the cation due to its unavailavility in industrial media. However, calcium is said to be least important to yeast growth and fermentation and its presence in fermentation media causes deleterious effects on the growth of yeast. It may also decrease the availability of other important cations like magnesium due to competition for binding sites. Zn^{2+} , on the other hand, is an important micronutrient and is present in most industrial media but is sometimes

unavailable for yeast due to binding with the complexing agents. However, excess concentrations of Zn^{2+} are known to affect the viability of *S.cerevisiae.* Further, according to the above discussions Mg^{2+} is a major cation in fermentative metabolism of yeast in synthetic media as well as in industrial media. According to previous studies Mg^{2+} exerts a protective effect on yeast from stress caused by temperature, osmotic pressure and ethanol toxicity. However, Mg^{2+} may become unavailable during fermentation of industrial media due to chelation, adhesion, or any other form of complexing or competition with other divalent cations like Ca^{2+}. Accrding to work by Maynard (1993), removal of Mg^{2+} from the growth medium by *S.cerevisiae* cells follows a regular pattern of uptake and release, the latter coinciding with the onset of the stationary phase of growth. This has led to the idea of Mg^{2+} -preconditioning yeast inocula prior to inoculation. However, very few published reports are available in this area of research and therefore in the present study it was decided to study some of the roles of Mg^{2+} ions in fermentation of industrial media and the relationship of Mg^{2+} with the other major important cations, K^{+}, Ca^{2+} and Zn^{2+}.

1.3.5 Molasses Fermentation Industry in Sri Lanka.

Fermentation production of potable alcohol is one of the oldest industries in Sri Lanka. With the rapid expansion of the sugar industry, the total molasses production has gone up and in 1995 it was about 40.000 metric tons. This production is expected to double within the next decade. Although this amount of molasses is adequate for the production of the domestic requirement of potable alcohol, Sri Lanka imports about 1/3 of her potable alcohol requirement. The main problem behind the incapability of production is the poor alcohol recoveries in distilleries. The average ethanol production per ton of molasses in Sri Lankan distilleries is about 300 proof liters, although, it is about 550 pl elswhere in the world (Patura, 1979). If the industry could achieve the above target there will be a big surplus of ethanol production and feasibility studies have already been undertaken to use ethanol as an alternate fuel to fossil oil. Therefore, it is clear that the economical potential and the requires improvements to the fermentation rocess. The

process. The major areas identified for the development are: selection and introduction of high yielding yeast strains and optimization of fermentation parameters; mainly yeast nutrients requirements, and temperature control in distilleries.

1.4 Objectives of this research

1. To asseses the applicability of controlling Mg^{2+} availability in industrial fermentation media for alcohol production by yeast.
2. To investigate the interactive effects of K^{+}, Mg^{2+}, Ca^{2+} and Zn^{2+} during yeast fermentation.
3. To evaluate the protective effects of elevated levels of extra and intracellular Mg^{2+} ions from ethanol toxicity in yeast.

CHAPTER 2: General Materials and Methods

2.1. Yeast cultures used for the experiments

Species	Code No	Original Source
Saccharomyces cerevisiae (Ditiller's strain)	DCL'M'	Quest international, Menstrie,UK.
Saccharomyces cerevisiae (Wine yeast)	L 2226	From Lallemond Inc Montreal, Canada.
Saccharomyces cerevisiae (Wine yeast)	L 2506	----------do----------
Saccharomyces cerevisiae (Brewer's ale yeast)	NCYC 1109	National Collection For Yeast Cultures, Norwich, UK.
Saccharomyces cerevisiae (Brewer's lager yeast)	NCYC 679	----------do----------
Saccharomyces cerevisiae (Baker's yeast)	DCL	From Quest International
Saccharomyces cerevisiae (Baker' yeast)	Mauri	From Mauri Yeast Products, Hull, UK.
Saccharomyces cerevisiae (Baker's yeast)	Red Star	Universal Food Co. USA.
Saccharomyces cerevisiae (Baker's yeast)	Saf-Levure	S. I. Lesaffre, France.
Schizosaccharomyces pombe	NCYC 1354	From National Collection for Yeast Cultures
Kluvyeromyces marxianus	NCYC 1425	--------do----------

One or several yeasts were chosen from the above according to the objectives and the design of the experiment .

2.1.1. Yeast culture maintainance

Freeze-dried culture ampoules were taken from the University of Abertay Dundee culture collection and resuscitated in malt extract yeast extract peptone glucose (MYPG) broth. Loopfuls of actively growing cells were then streaked on MYPG agar plates and incubated for 24 - 48 h at 30°C and single colonies from the actively growing cultures were then streaked on malt extract agar slants, incubated as above and stored at 4° C. Cultures prepared in this way were treated as stocks for the preparation of starter inocula or seed cultures throughout the study.

2.2. General experimental techniques

2.2.1. Microbiolgical procedures

General microbiological techniques and aseptic conditions were followed throughout the culture handling, inoculation, transferring, sampling and other related activities during experimentations. All experimental glassware was heat sterilised, media were steam sterilised (110°C/ 10min) and heat labile substances were filter sterilised prior to use.

2.2.2. De-ionising glassware

All glassware used for fermentation and analytical studies was deionised by soaking overnight in a 2%(v/v) nitric acid (AnalaR grade) solution followed by a wash with ultrapure water prior to a 0.1 M. EDTA wash to remove any nitrate and metal ion traces with a final three washes with ultra pure water to completion.

2.2.3. Experimental designs

All experiments were planned according to standard scientific design methods and were replicated at least twice and averages of duplicate readings were used in all comparisons. Further, Analysis of Variance (ANOVA) and other statistical data analytical techniques were used wherever appropriate

2.2.4. Preparation of lipid supplementation media

As lipid supplemetation was required for the anaerobic fermentation by yeast of defined media, a stock solution of ergosterol and oleic acid was prepared by adding 0.6g of ergosterol to 10ml of ethanol followed by 100ml of Tween 80, a source of oleic acid. The stock solution was then sonicated at 14 microns for 25 min in a sonicating water bath (Cam lab, Transonic T310). The solution was then steam sterilised for 15min at 121 °C and 15 psi (O'Neill, 1986).

2.2.5. Chemical analyses

Ethanol determinations

A Gas Chromatograph (G.C.) Helwett Pakard (model 5710 A) fitted with a Propac Q stainless column was used to determine the ethanol levels in experimental samples throughout. Oven temperature was 120°C, flame ionisation detector (FID) temperature 200°C, injector port temperature 200°C, carrier gas nitrogen (60ml/min) and hydrogen gas (15 ml/min) and air (27ml/min) was used for the flame. The G.C. was left to equilibrate at least 30min prior to any analysis to stabilise the flame and gas flow.

At least 1.5 ml samples from experimental fermentations were withdrawn into Eppendorf tubes and microcentrifuged at 13,000 r.p.m to remove yeast cells. Resulting supernatants (475µl) were used to determine the ethanol contents after mixing with absolute isopropanol (25µl) as an internal standard at a final concentration of 5%v/v of the sample. The supernatant samples were further diluted with de ionised water whenever a higher ethanol content was expected (over 8%v/v). At least 2 injections per sample were made and the average was taken as the ethanol concentration (%v/v) of the particular sample.

Metal ion analysis

Total concentrations of magnesium, calcium and zinc ions were determined using an Atomic Absorption Spectrophotometer (AAS) Perkin- Elmer, 1100 B which was pre-programmed for ion analysis. Potassium ions were determined by a Flame Photometer (Corning model 400).

For magnesium analysis, external standardization for the metal was done after aspirating a series of magnesium standards solutions standard (0.05, 0.1, 0.2, 0.3, 0.4, 0.5ppm) prepared by diluting a stock solution of magnesium chloride (1000ppm, Fisons). The resulting calibration curve was stored in a separate file and used for the analysis throughout with prior verification by aspirating fresh standards. The details of the set parameters are given in Appendix.1.

For calcium analysis, external standardization was done using a series of calcium standards (0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 1 ppm) prepared from a $Ca(NO_3)_2$ stock solution (1000 ppm, Aldrich Chemical Company). A prepared standard curve was stored as for Mg^{2+}. The operational conditions are given in Appendix 1.

For zinc analysis external standardization was accomplished using a standard (500 ppm) $ZnCl_2$ solution prepared as follows: Accurately weighed 0.5g of new zinc shots (16-30 mesh, Analar, Hopkin & William) was dissolved in 10 ml of 50%v/v HCl in a 1L volumetric flask and made up to volume with ultra pure (18MΩ) water (SYBRON/Barnsted, NANO pure II). The working standards of 0.025, 0.050, 0.075, 0.1 ppm were prepared by further diluting the stock solution. A stored calibration curve was used throughout the analysis as for other ions. The operational conditions were as in Appendix.2.

Potassium ion analysis was conducted using a Flame photometer (Corning model 400). Two calibration curves were prepared after recording absorption readings for the standards (0-10 ppm and 0-100 ppm) of KCl (AnalaR grade) in ultra pure water (Figs 2.1 and 2.2). Large standrad deviations in the graps could be due to the errors caused by repetition and the variations of indicator deflections caused by tubulent gas flow during the analysis. However, as the correlation coefficents between the average absorption values and the indicator deflections were maximum, the standrad curves were used for the estimation of potassium ions.

Analysis of fermentation media for K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}

Five milliliter samples from all media preparations were removed and centrifuged to discard any insoluble particles present and then desirably diluted. The samples were then mixed with a lanthanam chloride solution (0.1- 1%) in order to prevent any interference of aluminium and some anions (PO_4, SO_4) during the determination of above cations. Samples were then directly aspirated in to the AAS. The concentration of lanthanam chloride and the levels of dilutions of the media samples varied and adjusted whenever necessary. The analysis of the fermented samples followed the same procedure after microcentrifugation (13.000 r.p.m, 5min) to remove yeast cells.

Analysis of cellular K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}

At least 1 ml of fermented broth samples was withdrawn from the experimental fermentations into Eppendorf tubes and microcentrifuged for 5 minutes at 13.000 r.p.m . Supernatants were then removed carefully and cell pellets resuspended in ultra pure water, vortexed, microcentrifuged and supernatants discarded. The washings were repeated 4 times to remove any surface bound and interstitial cations. (The number of washes was decided after experimental analysis which is detailed below). After the final wash, cells were resuspended in 0.5 ml of ultra pure water, transferred into 10 ml plastic tubes (Nalgene), the Eppendorfs rinsed with 0.5 ml of ultra pure water followed by 1 ml of conc. H_2SO_4 (BDH). The tubes containing cells were heated to 100° C for 30 min. in a water bath. Upon completion the digested samples were diluted to 10 ml with ultra pure water. Samples were further diluted if required or directly aspirated into the AAS or flame photometer for the determination of Mg^{2+}, Ca^{2+} , Zn^{2+} , and K^+ as described above. By digesting and diluting yeast samples it was expected that all cations would be extracted from the cells into solution creating a homogeneous solution prior to analysis (Seiler, 1990).

Analysis of cations in cell wash

10 ml samples of fermentations were removed into deionised plastic tubes, centrifuged and washed 6 times with ultra pure water as previously described. All cell washed supernatants were separately collected in another set of deionized plastic tubes and used for ion analysis. Supernatant samples were diluted or aspirated into the AAS and Flame photometer and readings were recorded as follows (Table 2.1).

Table 2. 1 Cation concentrations in cell wash

Wash no.	Ion concentration(ppm)			
	K	Mg	Ca	Zn
1	20	3.34	16	0.43
2	6	0.38	3.2	0.017
3	1	0.01	1.1	0.004
4	0	00	00	00
5	0	00	00	00
6	0	00	00	00

According to the above data (Table 2.1) after the 4th wash none of the above ions were present in the wash (also see Fig.2.3). Therefore, all experimental cell samples were washed 4 times prior to analysis to ensure removal of extraneous and loosely-bound metal ions. Large standrad deviations may be due to the errose caused during repetition and sample preparations.

Determination of total sugars in molasses

For the preparation of raw molasses samples, 12.5 g sample of molasses was transferred into a 250 ml volumetric flask and 25 ml of lead acetate (50% w/v) was added (inorder to decolourised molasses) and mixed thoroughly before addition of ultra pure water to volume. The contents were then filtered and 100 ml of the filtrate transferred into a 500

ml volumetric flask to which 10 ml of pre- prepared de-leading solution (a mixture of 3% w/v potassium oxalate and 7%w/v disodium phosphate solutions) was added in order to precipitate the lead ions. The volume was made up to 500ml with ultra pure water. Mixed flask contents were then filtered and filtrates used for sugar analysis.

For the preparation of fermented molasses samples, 25 ml of fermented molasses was centrifuged and supernatants decanted into 250ml Erlenmeyer flasks prior to addition of 50 ml of ultra pure water and 5 ml lead acetate. Mixed solutions were then filtered. De-leading solution (2ml) was added into the filtrate and re-filtered (Payne 1968). The final filtrate was used for total sugar analysis.

The total sugar content of samples were determined by the phenol sulphuric acid method (Dubois et al 1956). 2 ml of the diluted samples of the above filtrates were pipetted into a boiling tube and 1ml of 5% phenol solution added. 5 ml of conc. H_2SO_4 was added rapidly into the tube, the stream of acid was directed against the liquid in order to obtain rapid heat production and good mixing. The tubes were left to stand for 10 min then placed in a 30°C water bath for 20min. Samples from the solution were withdrawn into plastic cuvettes and absorbance measured at 490 nm using a spectrophotometer (Cecil series 2). Blanks were prepared by substituting ultra pure water for the sugar solution. The amount of sugar was determined by referring to a pre-prepared standard curve (Fig 2.4). Higer standred deviations may caused by the erros during repetition. As the standred curve had a higher correlation coefficent, it was used to estimate the total sugar contents in experimental samples.

2.2.6. Determination of cell numbers

Total cell numbers

Total cell numbers were determined using a Coulter Counter (C.C.) Model D (Coulter Electronics, Luton, UK) fitted with a 100μm orifice probe. Random checks, using an Improved Neubaur Haemocytometer (Weber BS748) were taken to verify the accuracy of the C.C. Desirably diluted samples were further diluted in 20ml Isoton 11(9% NaCl plus azide) and sonicated for 4 min in a sonicating water bath (Camlab Transonic, model 310) in order to disperse any cell flocs which may cause erroneous enumerations.

Viable Cell numbers

Haeamocytometer counts of a 1:1 mixture of methylene blue citrate-stained yeast cells were taken in order to assess the viability of yeast whenever necessary. (Methylene blue is a redox indicator which reduces into leuco form by the viable cells due to the mitochondrial dehydrogenase activity in the presence of citrate buffer). The stain was prepared as follows: 10 mg methylene blue (BDH) dye dissolved in 10 ml of ultra pure water and 2 g of sodium citrate dihydrate (BDH) was added , stirred until dissolved and filtered. The filtrate was then transferred into a 100ml volumetric flask and made-up to volume (Institute of Brewing recommended methods of analysis, 1991).

Determination of yeast cell biomass

A drying and weighing balance (Metler CJ 16 moisture analyser) was used for the dry weight estimation of the experimental yeast cell samples. 10 ml samples were removed from the fermentations into clean and dry plastic tubes. The samples were then filtered through pre-weighed Whatman Glass micro fibre filters (4.7 cm) after fitting into a Whatman glass screen membrane holder. Carefully removed filters were then dried on an aluminium tray of the balance to take the dry weight.

Fig 2.1 Standard curve for potassium estimation(0-10ppm)

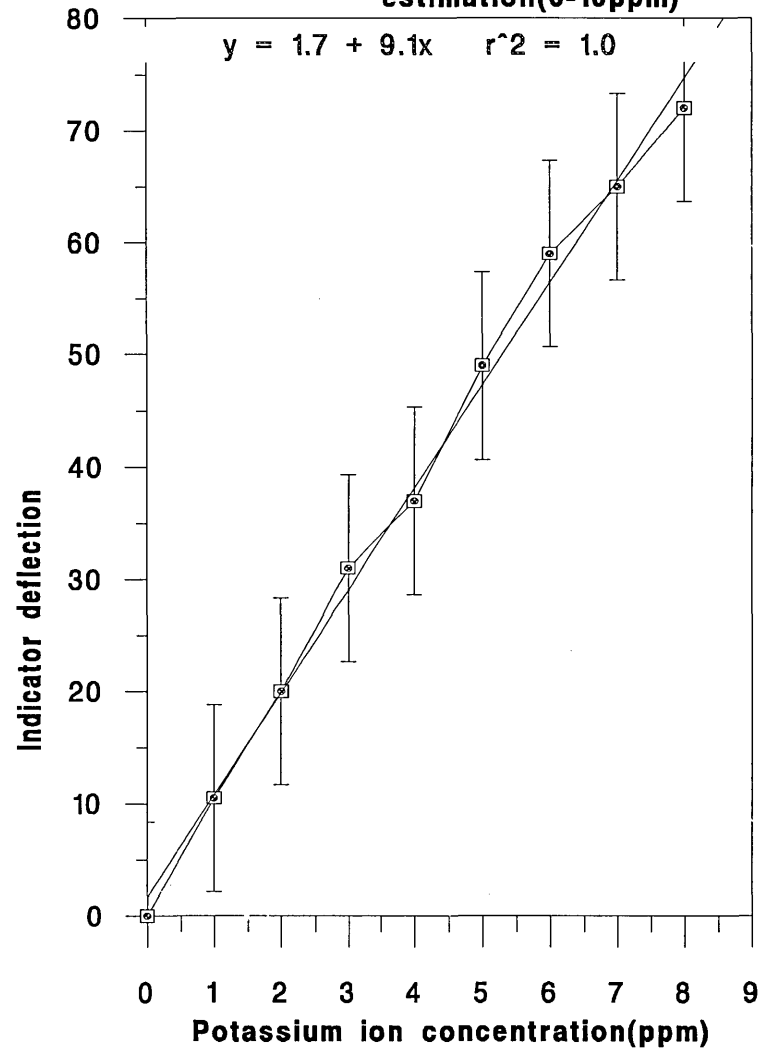


Fig 2.2 Standard curve for potassium estimation (10-100ppm)

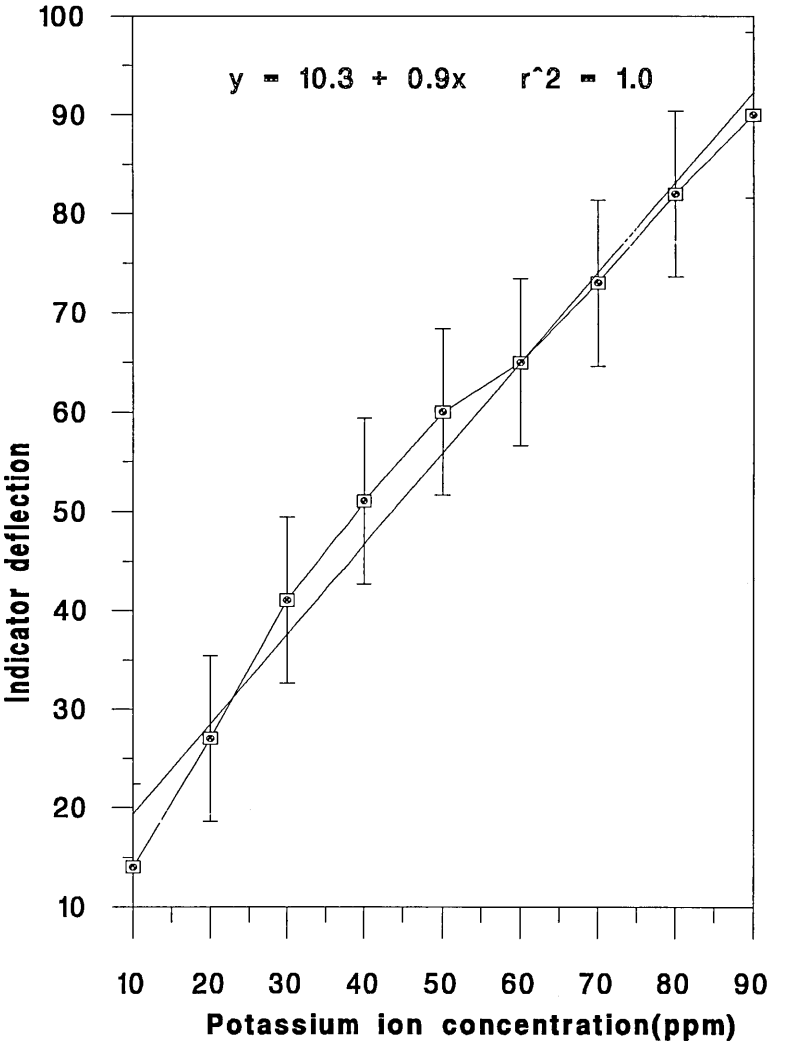


Fig 2.3 Influence of water-washing on removal of cations from cells

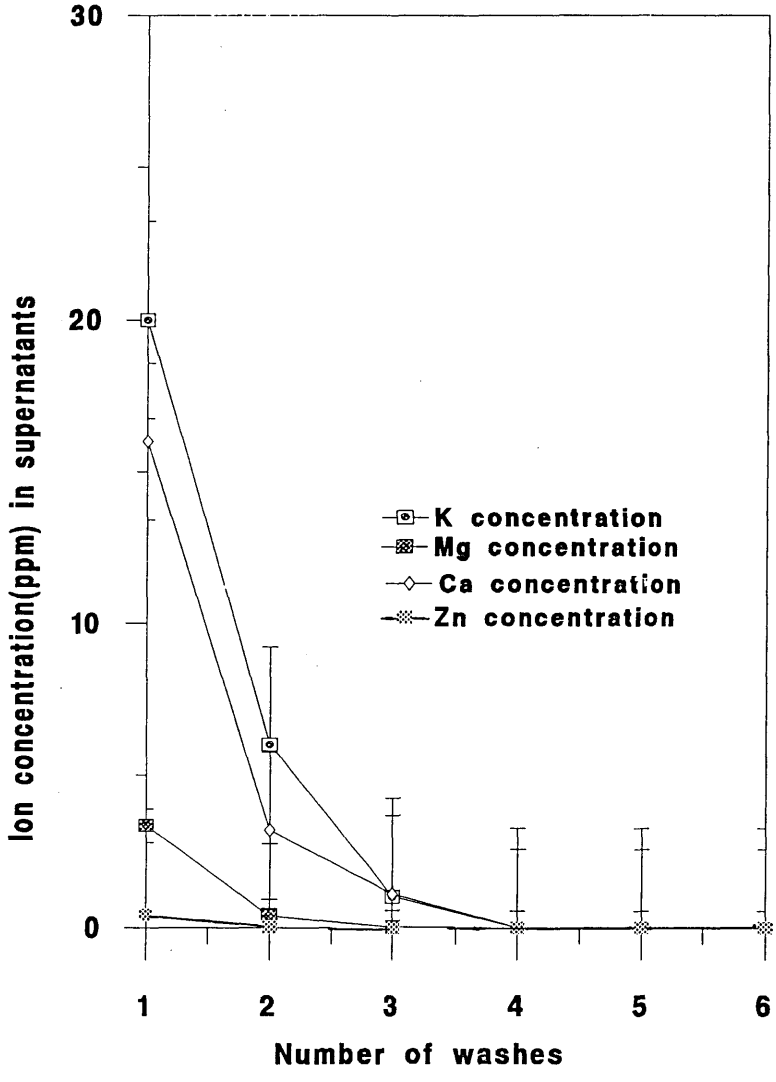
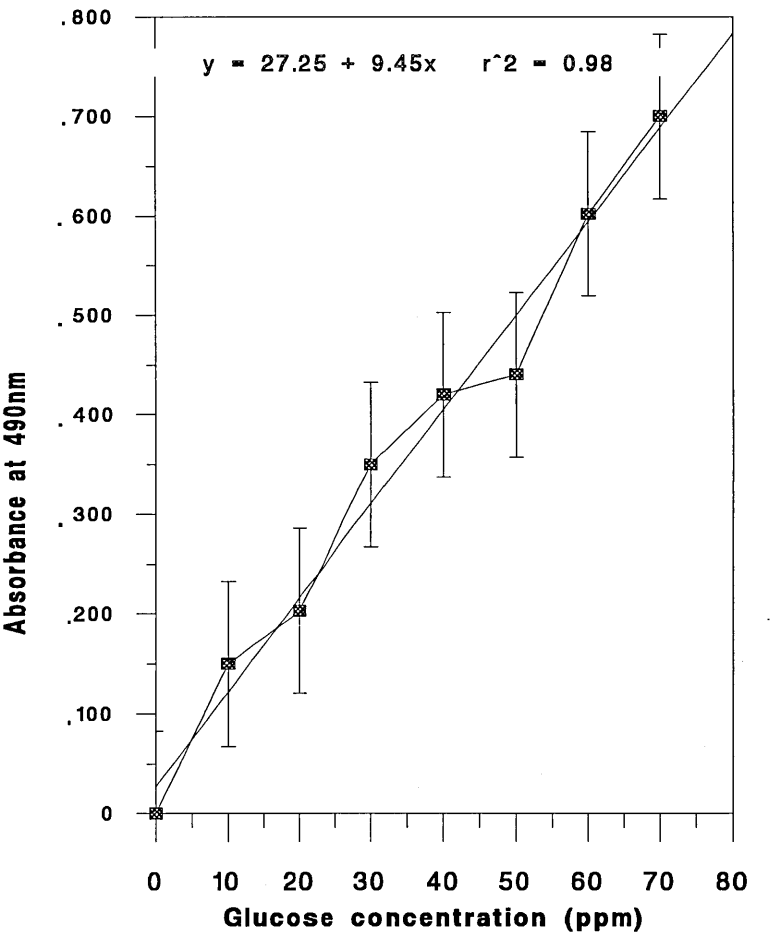


Fig 2.4 Standard curve for sugar estimation.



CHAPTER 3: Metal Ion Availability and Fermentative Metabolism in Yeast

3. 1 INTRODUCTION

Fermentation is a metabolic process characterised by incomplete oxidation and transformation of organic substances. It can be defined as a process in which chemical changes are brought about in an organic substrate through the action of biochemical catalysts called enzymes elaborated by specific types of yeast, fungi and bacteria. Although these organisms differ in their morphology and other characteristics they all produce enzymes which catalyse specific reactions ascribed to them. Other important factors, viz. temperature and pH influence the functions of these enzymes, as well as metallic co- factors like Mg^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+} which are essential in the transfer of electrons and protons from the substrates.

Ethanol is an aliphatic alcohol with many uses and has in recent years become synonymous with renewable energy. Its production, via fermentation of cheap carbohydrate products such as molasses, cassava starch and some abundant easily degradable cellulosic materials using low cost processes, to be used in internal combustion engines is a commercial reality. There is enormous potential for bioethanol in developing countries like Sri Lanka which depend on foreign fossil oil resources. Some countries have already acquired this technology and in the United States the product called "Gasohol" which is a 20:80 mixture of ethanol:petrol is employed in internal combustion engines successfully. Apart from the above uses ethyl alcohol has great economic value as an industrial raw material in the production of acetone, acetaldehyde and ethylene (which are also again used for the production of various chemical compounds) by oxidation, dehydration, or modification (Paturau, 1979). Further, alcoholic beverages (beer, whisky, wine, rum, sake) produced by fermentation of carbohydrates in malt wort, molasses, wine must, rice, maize etc. using yeast has a long history and to-day has developed as a major industrial process. These industries play major roles in the economies of many countries and use modern technologies to

improve efficiencies and the qualities of products. The function of yeast is vital to the fermentation process and in recent years tremendous efforts have been made to improve the alcohol production capability of yeasts which are used in industry (Harrison and Graham, 1967). Increase of conversion efficiencies of fermentation processes is vital for the economics of commercial ethanol production as distillation costs amount to more than 40% of overall costs. The percentage alcohol in the fermented wort is one of the major factors which determine the cost of distillation and therefore any attempt to increase it would definitely have great industrial significance.

Hence, the yeast cell acts as an individual production unit or a biocatalyst and the number of cells present in the fermentation medium is vital to maintain the efficiency of the process. Therefore, the role of yeast nutrients is vital in industrial fermentations. Brewer's and baker's yeast grow well in simple media containing fermentable sugars as a source of energy and carbon for biosyntheses together with an ammonium salt as a nitrogen source in the presence of mineral salts and growth factors (Cuts and Rainbow, 1950). According to Harrison and Graham (1967) most of the industrial feedstocks like molasses and malt wort contain an appreciable array of nutrients. However, temporal variability of the composition of the fermentation media is inherent in natural substances which greatly influences not only the fermentation process but also the productivity of ethanol (Oostsu, 1942, cited by Nagamune and Innone, 1981). Therefore, adding nutrients is necessary at least during the inoculum development stage. Several workers have shown that adding ammonium sulphate, magnesium sulphate, calcium chloride and yeast extract into molasses not only increases the rate of fermentation but also ethanol yields (Pandy and Agarwal, 1993). Further, Wong (1993; cited by Pandy and Agarwal, 1993) showed the importance of cations: potassium, magnesium , calcium , zinc, aluminium, iron, copper, cobalt and phosphate anion in ethanol fermentation. However, some of these cations were toxic when present in excess concentrations. Even though the importance of supplementation of mono and divalent cations in industrial fermentation media for proper yeast growth and metabolism have been emphasised by

several workers, very little attention has been paid to the practice in the belief that industrial media contain sufficient quantities of cations. However, working on malt wort, beet and cane molasses, Walker *et al* (1990) and Wolniewicz *et al* (1988) have shown that by adding the divalent cation, magnesium, to the fermentation media higher ethanol yields were achieved. Furthermore, calcium may exert inhibitory effects when present at higher concentrations. The positive effect of magnesium could be due to the fact that magnesium is an essential co-factor of more than 300 enzymes and acts in phosphorylation of sugars. Magnesium ions influence the glycolytic activity and mitochrondrial function of yeast (Walker *et al,* 1982, 1990). Recent work on the role of magnesium ions (Walker, 1994) has suggested that the fate of pyruvic acid is decided by the availability of magnesium ions in the medium resulting in higher fermentation rates when more Mg^{2+} is made available. Therefore, this study aimed to elucidate the influence of some cations on the fermentation performances of industrial yeast with special reference to magnesium.

3.2 Experimental Approach

A series of experiments were conducted to investigate the effect of magnesium ion supplementation on alcohol production by industrial strains of yeast, mainly in molasses media.

3.2.1. The performance of different yeast strains in Mg^{2+}- supplemented media

South African cane molasses and Yeast Extract Peptone Dextrose broth (YEPD) were fermented by the following commercially-available yeast strains: *S.cerevisiae* DCL'M' distillers' yeast (from Quest International, Menstrie), wine yeasts L 2256 and L 2056 (from Lallemand Inc, Montreal), brewer's ale yeast (NCYC 1109), lager yeast (NCYC 679), baker's yeast DCL (from Quest), *Schizosaccharomyces pombe* (NCYC 1345) and *Kluyveromyces marxianus* (NCYC 1254). Yeast cultures were grown on malt extract agar slopes and used for inocula preparations.

The seed molasses inoculum medium comprised: South African molasses (BP Chemicals, UK) (100g), $(NH_4)_2SO_4$ (2g), $(NH_4)_2PO_4$ (0.3g) and ultra pure water (18MΩ) to 1L. Initially, molasses was clarified as follows; 100g of molasses in a de-ionised 2L Erlenmeyer flask was dissolved in 1L of ultra pure water, adjusted the pH to 5.5 with sulphuric acid (50% v/v), pasteurised at 90 °C for 15 min and left overnight for sedimentation. To clarified molasses (200ml) separately sterilised nitrogen and phosphate sources were incorporated aseptically. Well mixed flasks were then inoculated with 48 hr washed suspensions of the above yeasts. Cultures were then incubated at 30°C in a Gallenkamp rotary incubator at 150 r.p.m. for 48 h. prior to harvesting by centrifugation. Cells were then resuspended in ultra pure water and used as the inoculum for experimental fermentations.

Production medium for molasses fermentation comprised: South African molasses (200g), sucrose (200g), $(NH_4)_2SO_4$ (2g), $(NH_4)_2$ PO_4 (0.3g), $MgSO_4.7H_2O$ (2g), 1L ultra pure water to completion.

Preparation of the production medium was as follows: initially molasses and sugar was clarified as previously by acidification, pasteurisation and sedimentation. Separately sterilised nitrogen and phosphate sources were then added to clarified molasses (100 ml samples in 250ml Earlenmeyer flasks). Sterilised $MgSO_4.7H_2O$ solution (to 200ppm) was then added leaving a flask without added magnesium as a control for each yeast strain. Flasks were then inoculated separately with yeast cultures previously grown as described earlier and harvested by washing and centrifugation, to get final cell numbers of approximately 10×10^6 /ml . All flasks were then incubated at 30°C in a rotary incubator (Gallenkamp) at 75 r.p.m.

The seed medium for YEPD fermentations comprised: yeast extract (Difco) (5g), mycological peptone (Difco) (5g), glucose (B.D.H) (20g) , ultra pure water to 1L. Initially 200 ml of sterilised above medium was inoculated with starter yeast cultures and flasks were incubated as previously described for molasses fermentations.

Production media comprised: yeast extract (5g), mycological peptone (5g), glucose (200g) to 1L . Initially these ingredients were weighed into a 2L flask and dissolved in 1L of ultra pure water by heating. 100 ml aliquots of the properly dissolved medium was then transferred into de-ionised 250 ml Erlenmeyer flasks and sterilised at 110°C for 10 min. Upon cooling, 200 ppm Mg^{2+} was added as previously. There were two flasks per each yeast strain leaving one without added Mg^{2+} as a control. All flasks were then inoculated with pre-grown starter inoculum as prepared previously, to get approximately 10×10^6 cells/ml. All flasks were then incubated with shaking at 30°C .

Sampling and ethanol analysis

Samples (1ml) from the fermenting broth were withdrawn aseptically at 12 hr intervals to estimate the kinetics of ethanol production. All samples were micro-centrifuged to remove cells before any analysis. Ethanol contents of all samples were analysed after preparing them as described under the Methods section (2.2.5), using Gas Chromatography. Results are summerised in Table 3.1, 3.2 and Figs 3.1- 3.4.

3.2.2. Performance of industrial yeast strains in high gravity industrial and laboratory media under different magnesium ion concentrations

The locally available distillers yeast DCL'M' strain (from Quest International, Menstrie), Red Star, Saf-levure baker's yeast (which are used in Sri Lankan distilleries) were evaluated for ethanol production in South African molasses, Sri Lankan molasses (Sugar cane Research Institute Sri Lanka), malt wort (Laboratory extracted from "Prisma" variety malt kindly supplied by United distillers, UK) and YEPD broth. Initially starter cultures were grown on malt extract agar and stored at 4°C. Fresh culture slopes were prepared on the same medium and incubated 48 h at 30°C prior to use as starter cultures for inoculum preparation.

Sri Lankan molasses medium comprised: Sri Lanka molasses (200g), sucrose (200g), $(NH_4)_2SO_4$ (2g), $(NH_4)_2$ PO_4 (0.3g), and variable levels of Mg^{2+}. South African

molasses media comprised: South African molasses (200g), sucrose (200g), $(NH_4)_2SO_4$ (2g), $(NH_4)_2 PO_4$ (0.3g), Mg (variable)

Yeast extract peptone dextrose broth comprised: Yeast extract (Difco) (5g), mycological peptone (5g), glucose (200g), Mg (Variable)

Malt extract broth prepared as follows: laboratory extraction was done by infusion mashing of 60g of Prisma variety malt grist in a flask containing 250 ml of ultra pure water for 70 min at 68°C in a water bath maintained at 71°C. The cooled mash was then centrifuged to separate the wort. The final S.G of the wort was 1062. It was then supplemented with $(NH_4)_2SO_4$ (2g), $(NH_4)_2 PO_4$ (0.3g), and variable quantities of Mg^{2+} as required.

As there were three yeast strains and five magnesium levels (150, 100, 50, 25 and 0 ppm) fifteen 250ml Erlenmeyer flasks were used for the study (5 per each strain). Initially Sri Lankan molasses solutions were acidified (pH 5.5) pasteurised and clarified as previously before addition of nitrogen and phosphate sources. Magnesium ions were added accordingly and flasks inoculated with washed yeast cultures at a cell density of 10×10^6 cells/ml. Cultures were incubated with shaking for 78h at 30°C.

Samples from the fermenting broth were withdrawn aseptically for cell numbers and ethanol analysis. Cell numbers were estimated using a Coulter counter. Ethanol was analysed as previously described. The above fermentation procedure was followed for the fermentation of all other media (South African molasses, YEPD and malt wort). Results are summerised in Table 3.3 and Figs 3.5 - 3.16.

3.2.3. Performance of DCL'M' in yeast in different types of Mg-supplemented molasses

The fermentation capability and Mg^{2+} uptake patterns during fermentation of Mg^{2+} - supplemented Sri Lankan (kindly supplied by Sugarcane Research Institute Sri Lanka), South African, Javan, Brazilian (kindly supplied by BP Chemicals, UK) Mexican, Pakistan (kindly supplied by Intermalt, UK) cane molasses and Danish beet molasses (kindly supplied by Lallemand Inc, Montreal) by the yeast *S.cerevisiae* DCL'M' strain were evaluated.

For each molasses, four concentrations (100, 200, 250, 300g/L) were chosen for evaluation. Initially molasses solutions were prepared in 2L Erlenmeyer flasks by dissolving each molasses in desirable quantities of ultra pure water and acidified (pH 5.5) sterilised and clarified as previously described. 100ml aliquots were then removed into 250 Erlenmeyer flasks and sterilised $(NH_4)_2SO_4$ (2g), $(NH_4)_2PO_4$ (0.3g), and 100ppm Mg^{2+} ions added leaving Mg^{2+} unsupplemented flask as a control. Flasks were then inoculated with a 48h culture of the yeast *S.cerevisiae* (DCL'M' strain) grown in $(NH_4)_2SO_4$ (2g), $(NH_4)_2$ PO_4 (0.3g) supplemented 10%(w/v) Sri Lankan Cane molasses. The final cell concentration was approximately 10×10^6/ ml. Flasks were then incubated at 30°C in a rotary incubator at 75 r.p.m for 78h. Similar experimental procedures were followed for the fermentation of all other molasses.

Samples from the fermenting broth were withdrawn aseptically at 6, 12, 18, 24, 30, 42, 54, 66, and 78 h intervals for cell counts and ethanol determinations. Samples were analysed as previously described. Cell magnesium were analysed in by AAS after digestion of the samples as described in the Methods section (2.2.5). Results are summerised in Table 3.4 and Figs 3.17 - 3.27.

Uptake patterns of magnesium into yeast cells (DCL'M' strain) during the early hours of fermentation was studied by withdrawing the samples at hourly intervals from a

fermenting Sri Lankan molasses broth which was supplemented with 100ppm magnesium.

Seed medium comprised: Sri Lankan molasses (100g), $(NH_4)_2SO_4$ (2g), $(NH_4)_2PO_4$ (0.3) and ultra pure water (18MΩ) to 1L. Initially molasses medium was sterilised and clarified and nutrients were added as previously and 100 ml of the clarified molasses decanted into a 250ml Erlenmeyer flask and inoculated with a suspension of the yeast DCL'M' previously grown on malt extract agar slant. The flask was then incubated at 30°C in a rotary incubator and final inoculum was prepared as for previous experiments .

Production medium comprised: Sri Lanka molasses (350g), $(NH_4)_2SO_4$ (2g), $(NH_4)_2PO_4$ (0.3) and ultra pure water to 1L.

Initially molasses was weighed into a 1L Erlenmeyer flask, acidified to pH 5.5, heat sterilised at 110°C and left overnight for clarification. 150 ml aliquots were then removed into two 500 ml Erlenmeyer flasks and sterilised nitrogen and phosphate solutions were added. Following the addition of nutrients one flask was supplemented with 100 ppm magnesium ions. Both the flask contents were then inoculated with a washed culture suspension of inoculum (DCL'M') to get a final cell density of approximately 10×10^6 cells/ml. Flasks were then incubated at 30°C in a rotary incubator with 75 r.p.m. The experiment was replicated.

Samples from the fermenting broth were removed at hourly intervals starting at 0 time up to 24 h and further samples were taken at 48 hr and 75 h of fermentation.

Samples were then analysed for cell numbers (Coulter Counter), ethanol (Gas Charomatography), residual sugars (phenol sulphuric acid method of Dubois et al, 1956), cell magnesium (AAS) as previously described (see Methods). Results are summerised in Figs 3.28 - 3.31.

3.3. RESULTS

Influence of Mg2+ on growth and fermentation of selected yeasts

Results of experiments conducted to investigate the fermentation performances of various industrial strains of *S.cerevisiae*, together with *Schizosaccharomyces pombe* (NCYC 1354), and *Kluyveromyces marxianus* (NCYC 2415) in South African molasses media and YEPD broth supplemented with magnesium are shown in Tables 3.1, 3.2 and Figs 3.1-3.4. The results revealed that a general increase in ethanol production occurred in all Mg^{2+}-supplemented flasks compared with the unsupplemented controls. These observations were irrespective of the types of yeast studied. In South African molasses, the *S.cerevisiae* distiller's DCL'M' strain showed an increase in rate of ethanol production after 24 h together with a prolonged exponential growth phase. The final ethanol concentration was higher (1.26%v/v) than the control (Table 3.1 and Fig 3.1). The fermentation kinetics of the wine yeasts L2056 and L 2226 are shown in Fig 3.2. Their responses to added Mg^{2+} in South African molasses were slightly different at the initial stage of fermentation. However, the final ethanol concentrations were higher in Mg^{2+} -supplemented media in both yeast strains (Table 3.1). The response of the lager yeast (NCYC 679) and the ale yeast (NCYC 1109) to added Mg^{2+} was not as prominent as the above yeasts. Nevertheless, both yeasts showed slight stimulatory effects on both ethanol production and kinetics of fermentation. This is clearly seen in Table 3.1 and Figs 3.2, respectively. The influence of Mg^{2+} on the fermentative metabolism of baker's yeast strain DCL was interesting since this strain showed a very high stimulatory response to added Mg^{2+} throughout the fermentation process by prolonging the exponential phase as well as producing higher ethanol concentrations at the end of the fermentation period. The yeast *S.pombe* (NCYC 1354) also showed a positive response to added Mg^{2+} in molasses by increasing ethanol yields (Table 3.1). Fermentation experiments conducted using the low ethanol tolerant, *K.marxianus,* showed a very high response to added Mg^{2+} by increasing the exponential growth phase and the final ethanol yield (Table 3.1 and Fig 3.1).

The response of the above yeasts in YEPD was somewhat different compared with molasses fermentations. For example, DCL'M' yeast responded poorly to added Mg^{2+} in YEPD broth (Table 3.2, Fig 3.3), whereas the wine yeasts L2056 and L2226 showed very high response to added Mg^{2+} , as in molasses (Table 3.2, Figs 3.3). Although the response of lager (NCYC 679) and ale (NCYC 1109) yeast to added Mg^{2+} in molasses was poor, in YEPD ale yeast produced a significantly higher ethanol content (an additional 2.1%v/v) than the control (Table 3.2). Although the baker's yeast (DCL) did not show a positive response during the early hours of YEPD fermentation, Mg^{2+} did extend the exponential phase and increase the final ethanol concentration (Table 3.2, Fig 3.4). Finally, *S.pombe* (NCYC 1354) was observed to exhibit a long lag phase in YEPD but nevertheless, Mg^{2+} prolonged the exponential growth phase and increased fermentation rate (Table 3.2, Fig3.4).

Table 3.1 Fermentation performances of different yeasts in South African molasses medium (fermentations were carried out at 30°C for 72h and Mg^{2+} -supplementations were at 100ppm).

YEAST	Alcohol concentration (%v/v)		Difference
	+Mg	-Mg	
DCL'M'	12.53	11.27	+1.26
L 2056 (wine)	13.08	11.30	+1.78
S.pombe	10.95	9.15	+1.80
K.marxianus	3.97	2.70	+1.27
NCYC 1109 (ale)	9.95	9.50	+0.45
NCYC 679(lager)	9.10	8.20	+0.90
Baker's (DCL)	11.30	10.00	+1.30

Table 3.2 Fermentation performances of different yeasts in YEPD broth (conditions as per Table 3.1)

YEAST	Alcohol concentration (%v/v)		Difference
	+Mg	-Mg	
DCL'M'	10.00	9.60	+0.40
L 2056 (wine)	10.99	9.31	+1.68
S.pombe	9.83	8.63	+1.20
K.marxianus	7.00	6.00	+1.00
L 2226 (wine)	11.60	9.20	+2.43
NCYC 1109 (ale)	10.90	8.80	+2.10
NCYC 679 (lager)	9.60	9.20	+0.40
Baker,s (DCL) yeast	10.6	9.20	+1.40

Table 3.3 Average values of ethanol production(%v/v) by some industrial yeasts in different media supplemented with varying levels of Mg

Growth media	Mg (ppm)	Type of yeast			Difference in ethanol production (Final - control)		
		DCL'M'	R.S	SAF	DCL'M'	R.S	SAF
SLM	1266*	11.00	11.00	9.19			
	1281	10.93	10.99	9.11	0.06	0.01	0.07
	1316	11.95	12.27	10.96	0.95	1.27	1.77
	1366	13.57	13.11	10.49	2.57	2.11	1.30
	1416	11.99	13.33	10.67	0.99	2.33	1.48
SAM	2400*	12.20	11.00	10.12			
	2415	13.20	10.53	10.48	1.00	0.46	0.36
	2450	12.96	13.14	10.90	0.76	2.14	0.78
	2500	12.98	11.98	11.08	0.78	0.98	0.96
	2550	13.41	11.75	11.12	1.21	0.75	1.00
YEPD	950*	8.10	9.10	9.26			
	965	8.92	9.11	9.32	0.82	0.11	0.05
	1000	8.55	10.0	9.51	0.45	1.00	0.24
	1050	9.09	9.99	9.70	0.99	0.99	0.43
	1100	9.31	10.00	9.99	1.21	1.00	0.73
M.wort	84*	6.40	5.70	4.99			
	99	6.95	5.11	5.01	0.55	0.59	0.02
	134	7.91	5.99	5.99	1.50	0.29	1.00
	184	7.95	5.81	5.82	1.54	0.10	0.82
	234	7.99	7.83	5.98	1.59	2.12	0.99

SLM = Sri Lankan mclasses, SAM = South African molasses, YEPD = Yeast extract malt exract peptone dextrose, M.wort = malt wort, R.S = Red Star, SAF = Saf- Levure. * = Control.

Table 3. 4 Average ethanol values following fermentations different in concentrations of molasses by DCL'M' yeast

	Molasses concentration (%w/v)							
	10		20		25		30	
	+Mg	-Mg	+Mg	-Mg	+Mg	-Mg	+Mg	-Mg
BM	4.00	3.56	6.26	5.87	9.25	7.85	8.37	7.00
BRM	4.00	3.19	6.80	5.68	7.50	6.69	8.80	7.89
JVM	3.29	3.14	5.76	5.34	7.62	7.28	8.66	7.90
MM	3.53	3.25	7.47	6.55	8.25	7.72	9.52	8.84
PM	3.30	3.09	5.77	5.43	7.52	6.80	8.66	7.94
SAM	3.06	2.90	5.54	5.54	6.94	6.53	8.70	8.03
SLM	3.60	3.32	7.44	6.75	8.75	7.86	9.00	8.54

+ Mg = 100ppm Mg added, - Mg = No added Mg

BM = beet molasses, BRM = Brazilian molasses, JVM = Javan molasses, MM = Mexican molasses, PM = Pakistani molasses, SAM = South African molasses, SLM = Sri Lankan molasses

Fig 3.1 Effect of Mg (100ppm) on ethanol production by *Kluyveromyces marxianus, Schizisaccharomyces pombe, Saccharomyces cerevisiae* (DCL baker's yeast and DCL'M' distiller's yeast) in South African molasses.

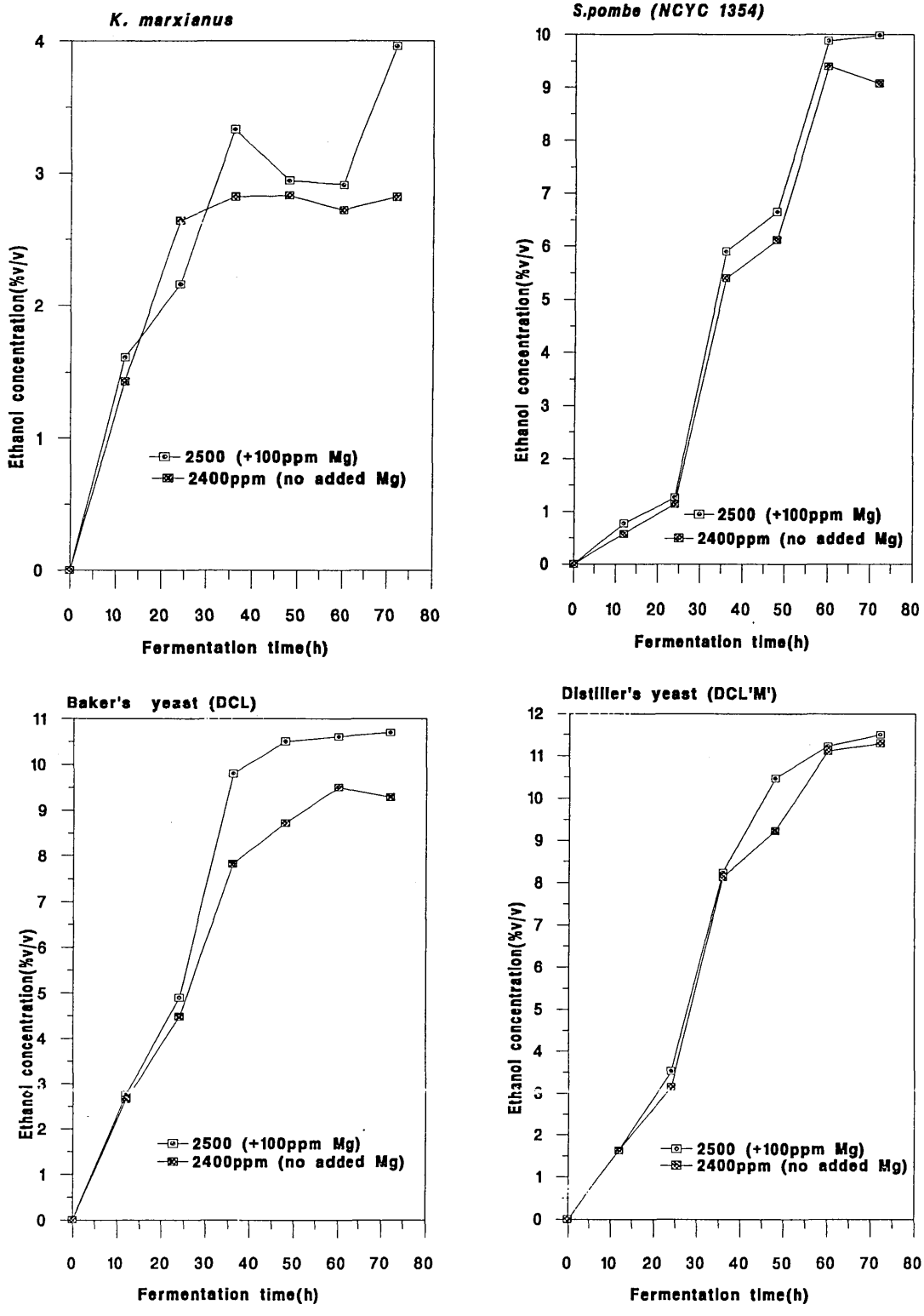
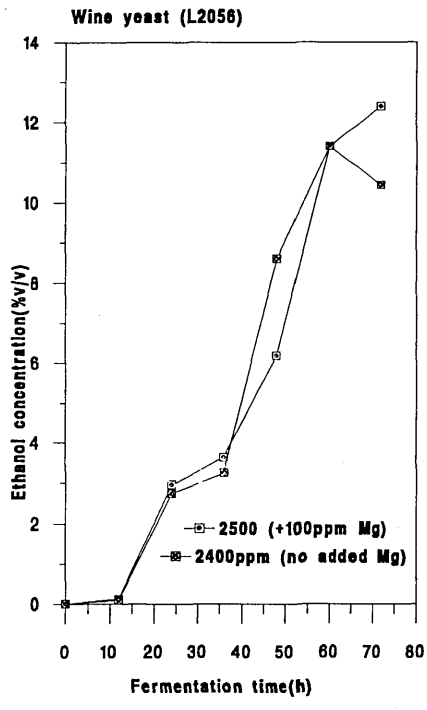
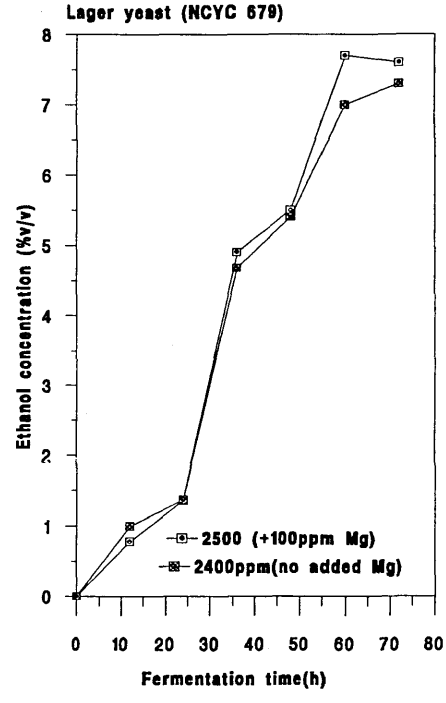
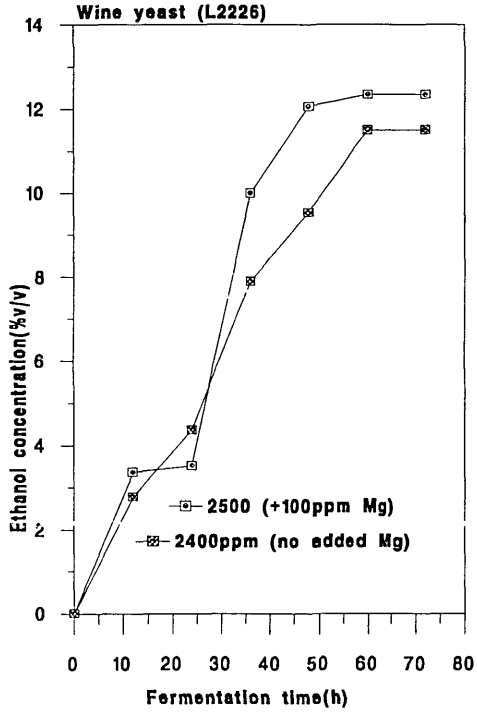


Fig 3.2 Effect of Mg (100ppm) on ethanol production by *Saccharomyces cerevisiae* wine yeast (L2056, L2226), Lager yeast NCYC 679 and Ale yeast NCYC 1109 in South African molasses.







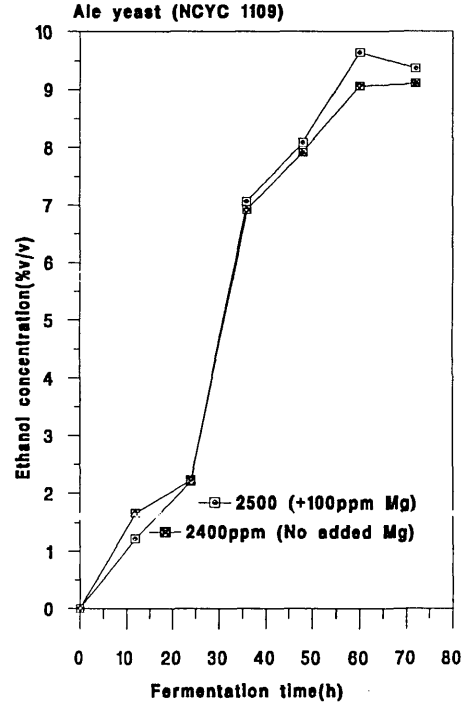


Fig 3.3 Effect of Mg (100ppm) on ethanol production by yeast *Saccharomyces cerevisiae* (DCL'M' distiller's yeast, Lager yeast, NCYC 679 and wine yeasts, L2226 and L2056 in Yeast extract Peptone Dextrose broth.

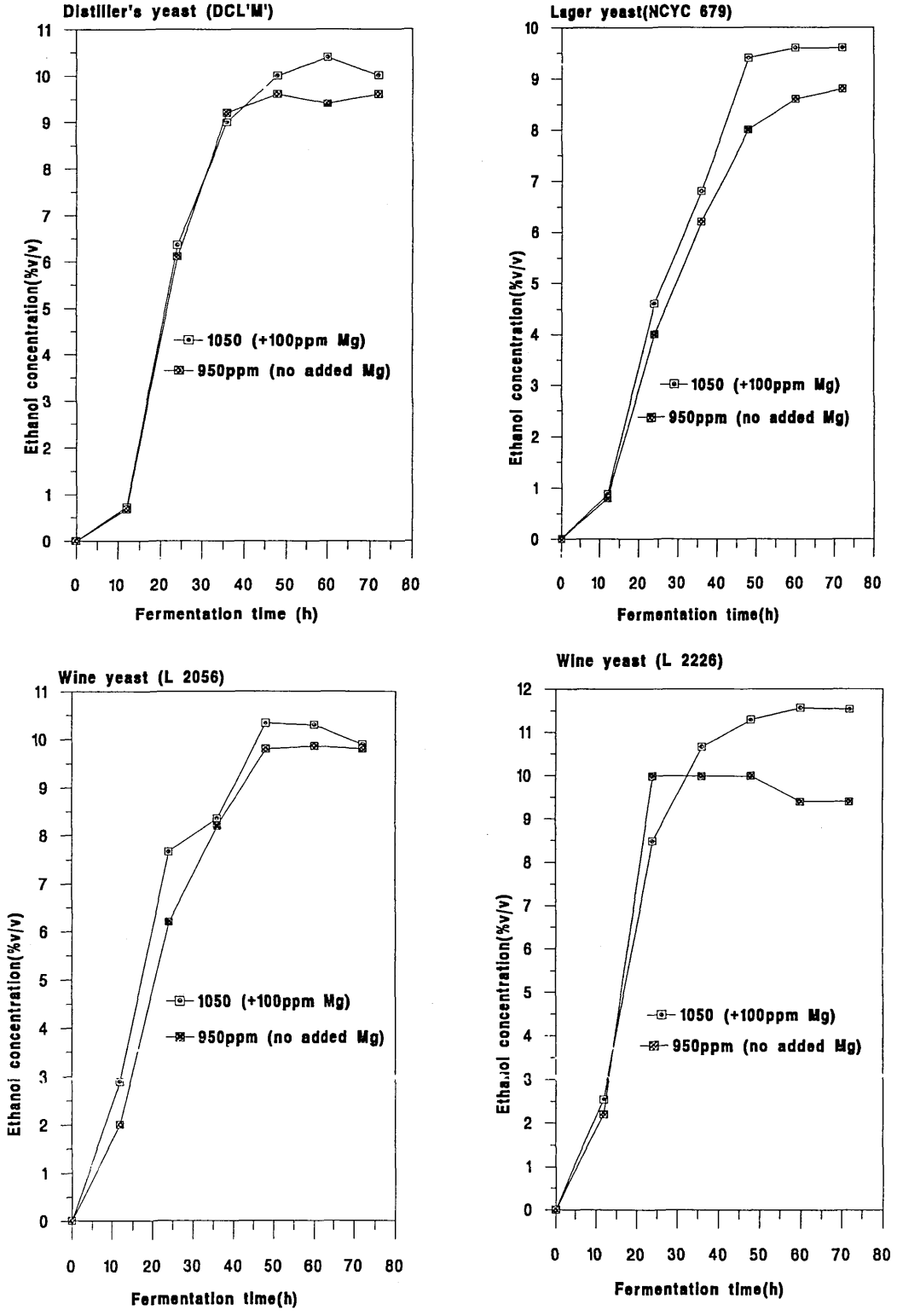
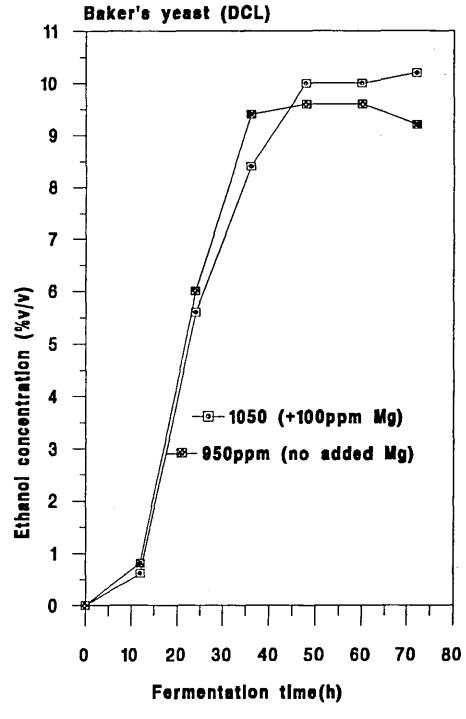
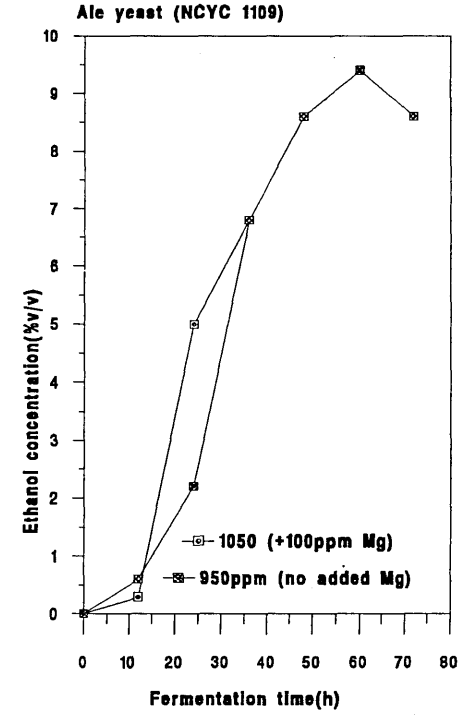
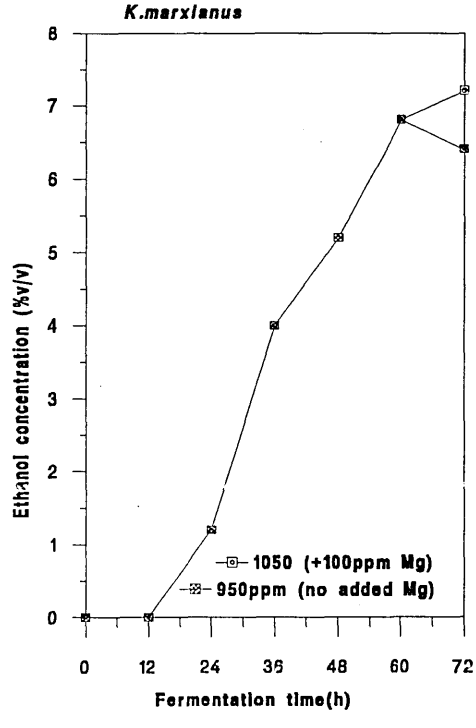
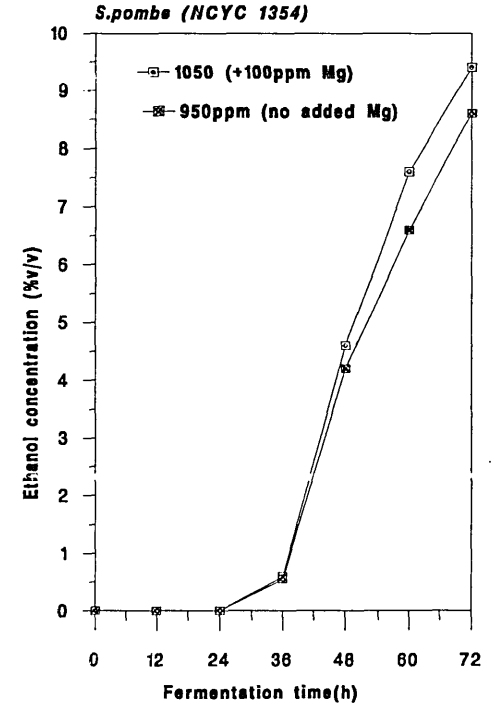


Fig 3.4 Effect of Mg (100ppm) on ethanol production by yeast *Saccharomyces cerevisiae* (DCL, baker's yeast, Ale yeast NCYC 1109, *Kluyveromyce marxianus* and *Schizosaccharomyces pombe* in Yeast extract Peptone Dextrose broth.









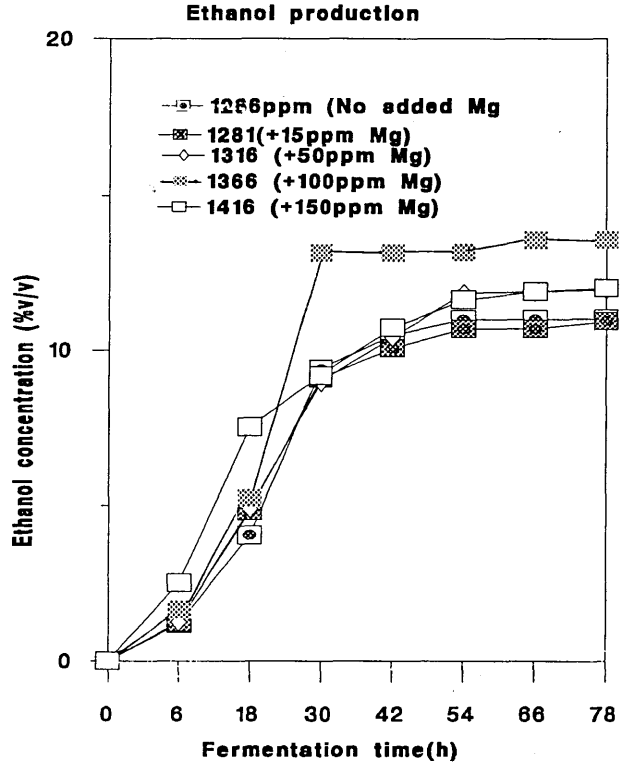
Influence of Mg2+ on growth and fermentation of selected yeast in high gravity media.

Results of experiments conducted to evaluate the performance of several commercially available yeasts viz. DCL'M'distiller's , Red Star baker's and Saf-Levure baker's yeast by fermenting Sri Lankan and South African molasses, YEPD broth and malt wort supplemented with 15, 50, 100, and 150 ppm Mg^{2+} are shown in Table 3.3 and Figs 3.5-3.16. During the fermentations, kinetics of cell multiplication and ethanol production were monitored. The objective of these experiments was to comparatively evaluate the performance of those yeasts in commercially employed industrial media like molasses and malt wort.

a) **Molasses fermentations**

Although Red Star and Saf-levure are baking strains, those are the main yeasts used in Sri Lanka for fermentation of sugarcane molasses. Present studies show a common stimulation of cell growth and ethanol production in all fermentation media with the increasing levels of Mg^{2+}. In Sri Lankan molasses DCL'M' strain produced the highest number of cells and ethanol concentration when media was supplemented with 50 and 100 ppm Mg^{2+}, respectively (Table 3.3, Fig 3.5). Cell multiplication of Red Star yeast in the presence of additional Mg^{2+} in Sri lankan molasses was higher than the unsupplemented controls with 100ppm Mg^{2+} being the most effective stimulatory concentration. For ethanol production, 150ppm Mg^{2+} appeared to be the most effective concentration (Table3.3, Fig 3.6). Saf-Levure baker's yeast also showed a similar response but final cell density achived was lower than DCL'M' or Red star yeasts. However, ethanol production by Saf-Levure increased in response to added Mg^{2+} towards the end of fermentation (Table 3.3, Fig 3.7). In South African molasses, DCL'M' yeast responded to added Mg^{2+} by increasing both cell number and final ethanol concentration (Table 3.3, Fig 3.8). The Red Star strain baker's yeast in Mg^{2+}-supplemented South African molasses also showed stimulatory effects as evidenced by

Fig 3.5 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in Sri Lanka molasses.



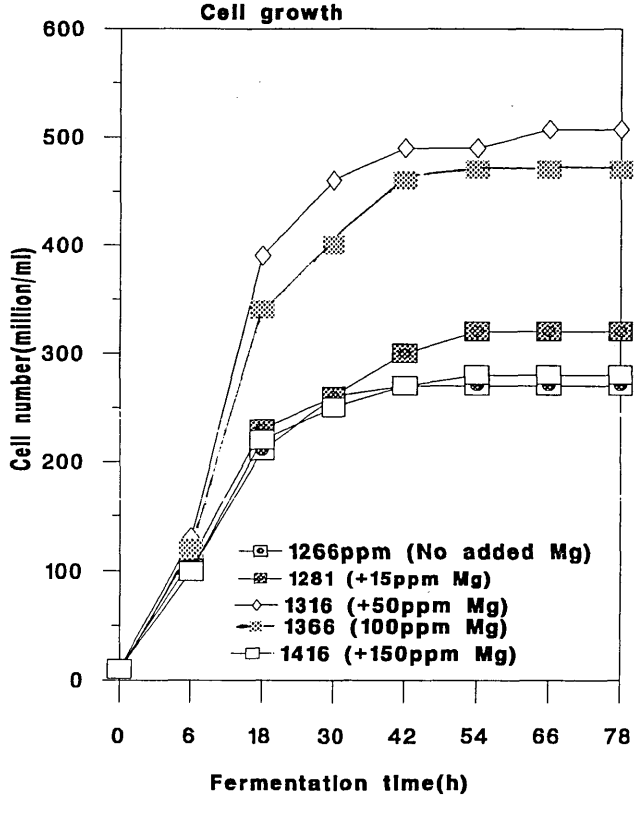


Fig 3.6 Effect of varying concentrations of Mg on cell growth and ethano production by *Saccharomyces cerevisiae* (Red star baker's yeast) in Sri Lankan molasses.

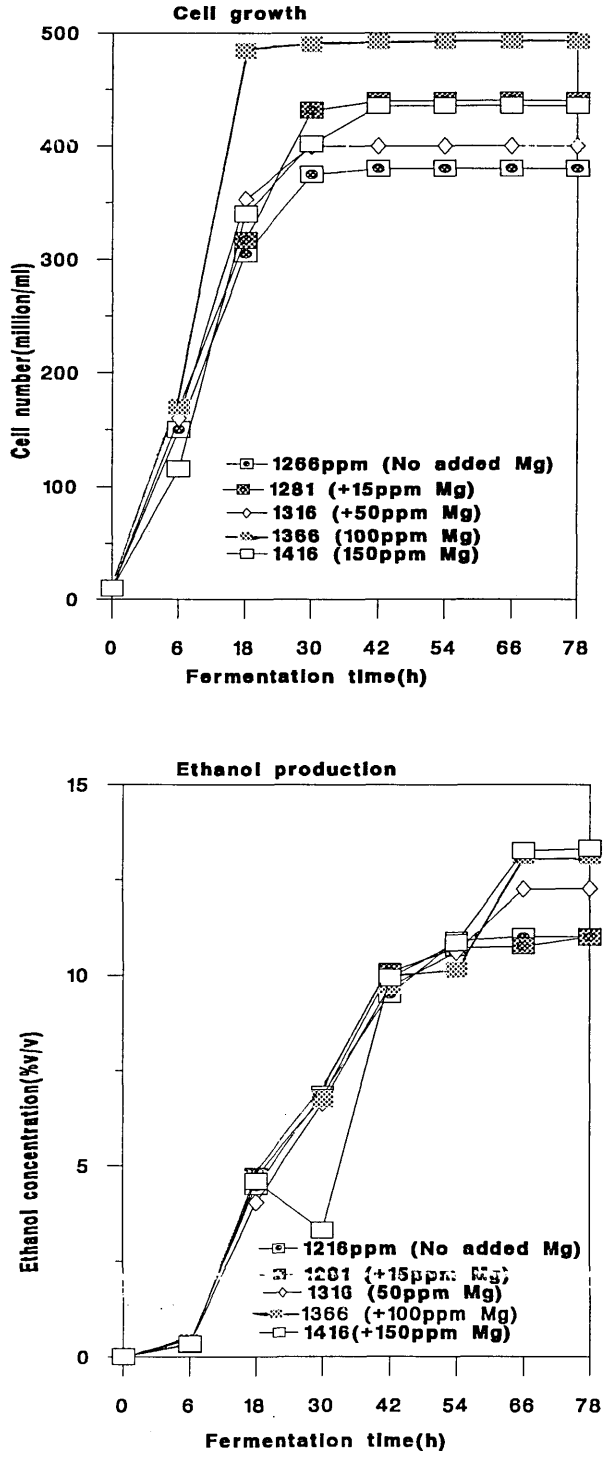
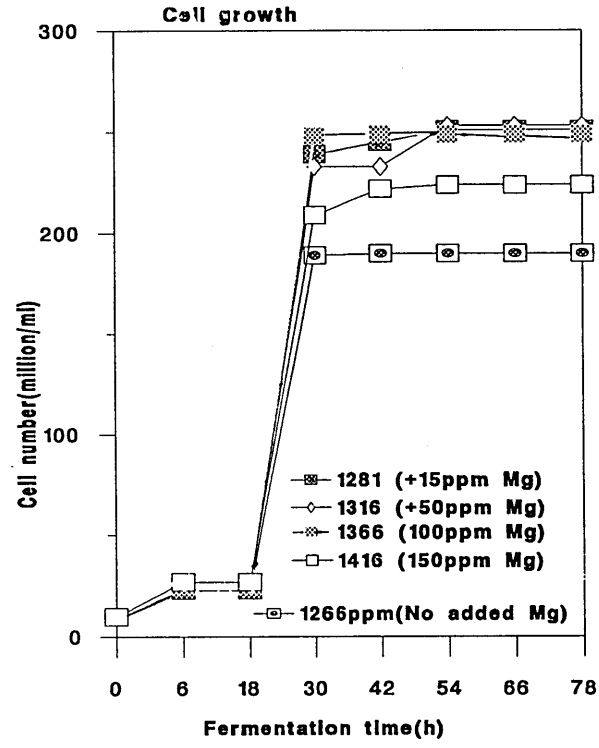


Fig 3.7 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (Saf-Levure baker's yeast) in Sri Lankan molasses.



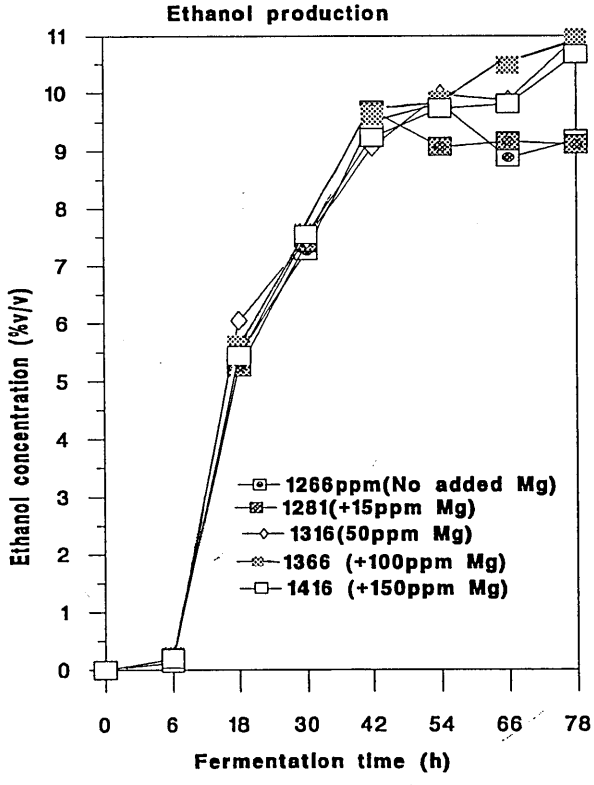
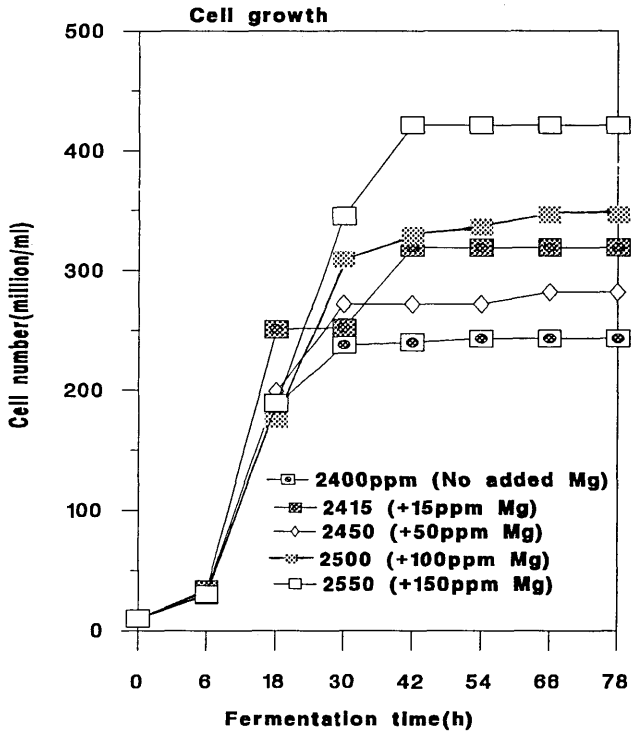


Fig 3.8 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in South African molasses.



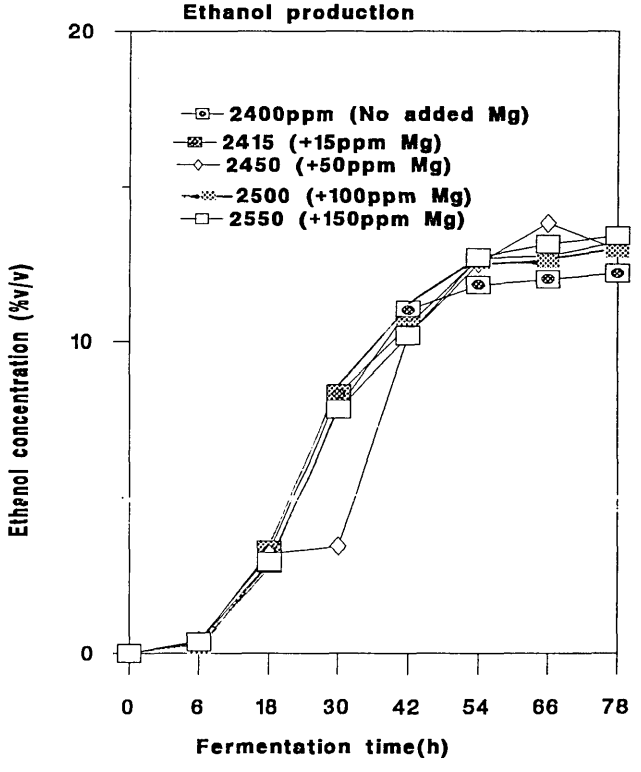


Fig 3.9 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (Red Star baker's yeast) in South African molasses.

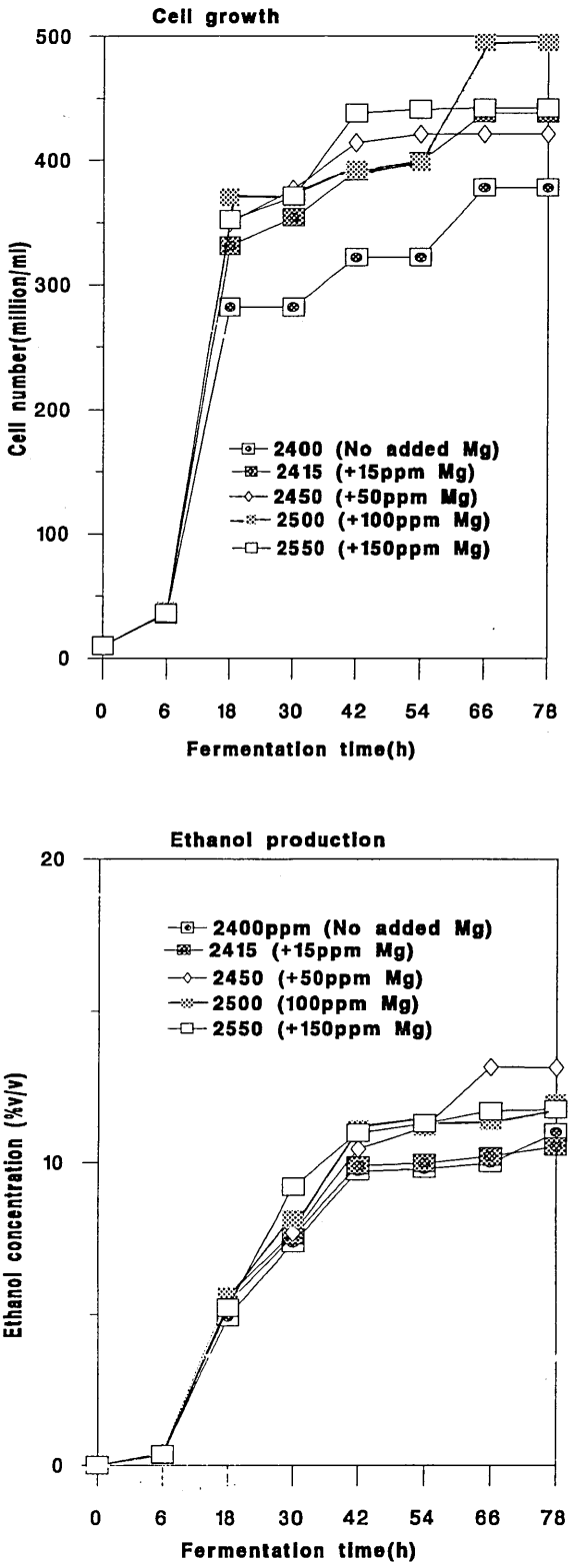
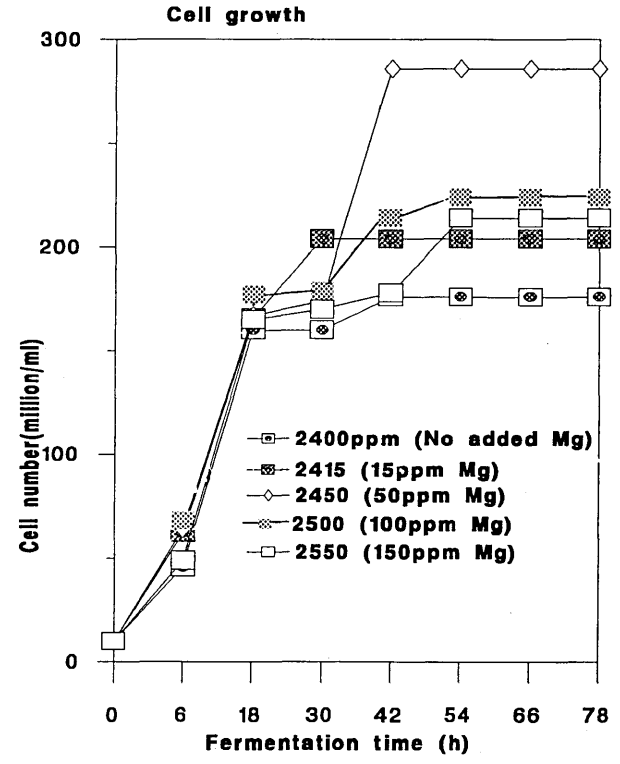
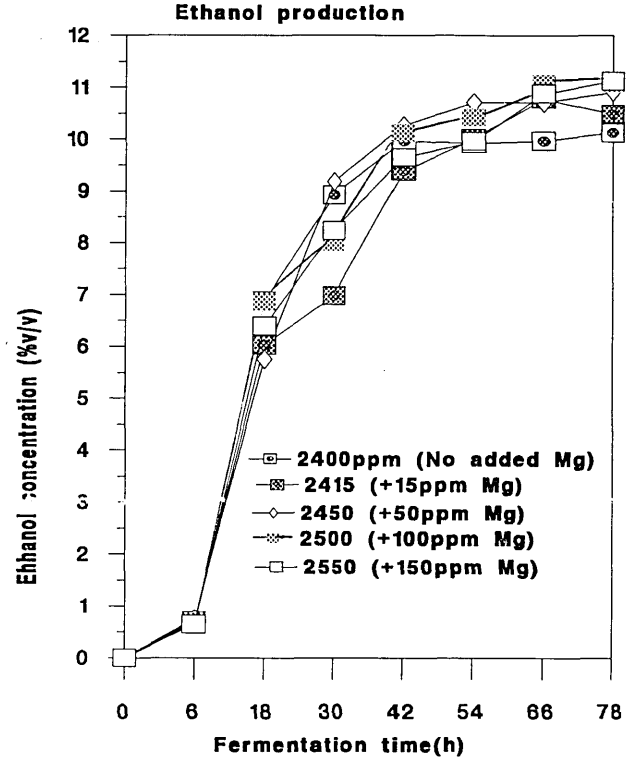


Fig 3.10 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (Saf-Levure baker's yeast) in South African molasses.



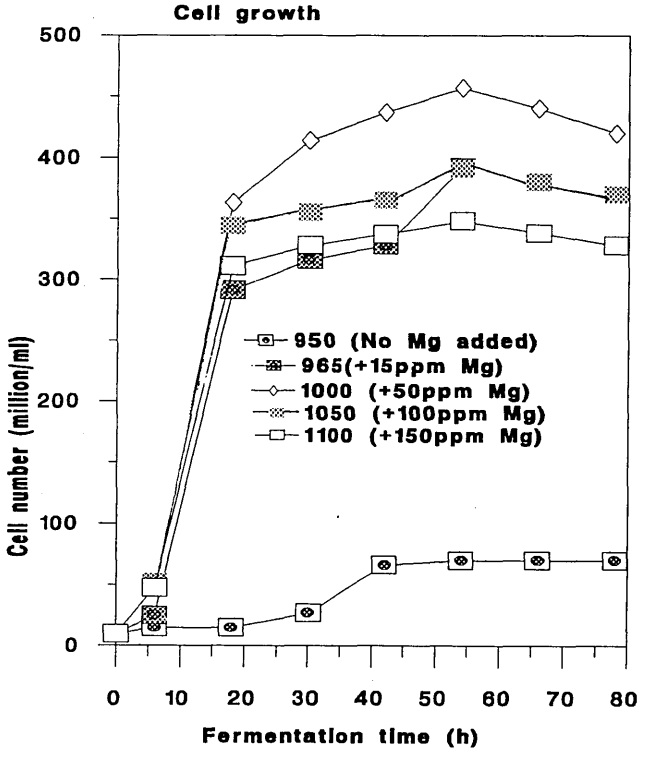


enhanced ethanol production and prolonged exponential growth phases (Table 3.3, Fig 3.9). The other baker's yeast strain, Saf-Levure, in Mg^{2+}-supplemented South African molasses similarly showed increase in cell number and ethanol concentration with increasing Mg^{2+} levels. (Table 3.3, Fig 3.10).

b) YEPD fermentation

In YEPD broth, DCL'M' growth and fermentation responded differently to Mg2+ supplementations compared with molasses. For example, growth during the early exponential phase differed and the highest ethanol concentration were recorded when media was supplemented with 150ppm Mg^{2+} (Table 3.3, Fig 3.11). Red Star baker's yeast showed rapid cell multiplication during the first 30 h of fermentation in the presence of additional Mg^{2+} in the medium. Furthermore, the fermentation performances increased with increasing Mg^{2+} concentration (Table 3.3, Fig 3.12). The yeast Saf-Levure in Mg^{2+} -supplemented YEPD broth again demonstrated a positive response toward added Mg^{2+} increased cell multiplication and ethanol production during the 78 hr fermentation period. (Table 3.3, Fig 3.13).

Fig 3.11 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in Yeast extract Peptone Dextrose broth.



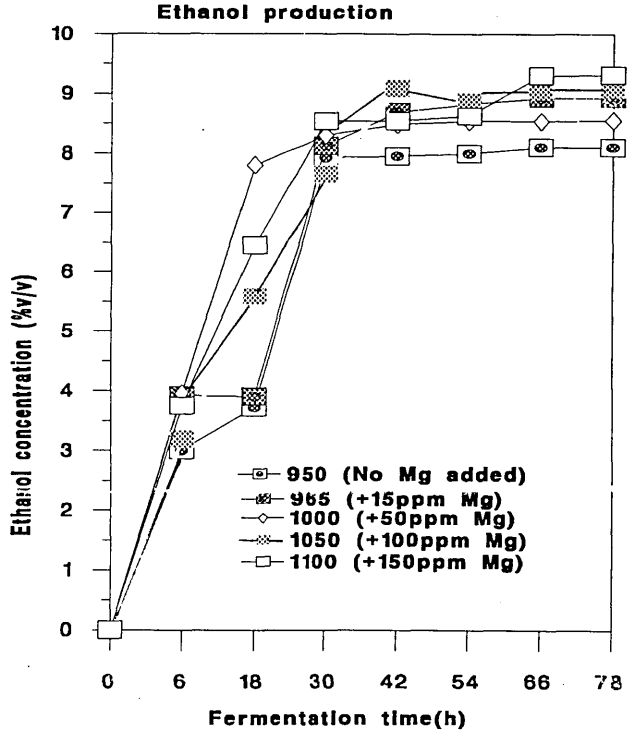
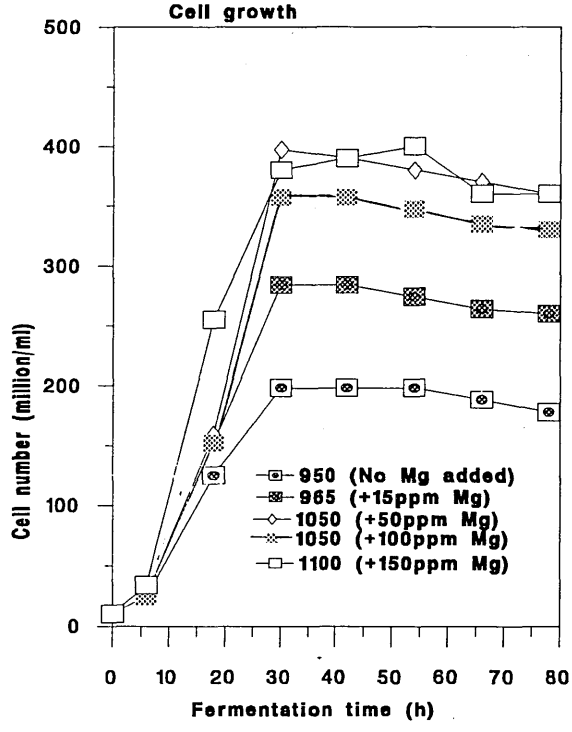


Fig 3.12 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (Red Star baker's yeast) in Yeast extract Peptone Dextrose broth.



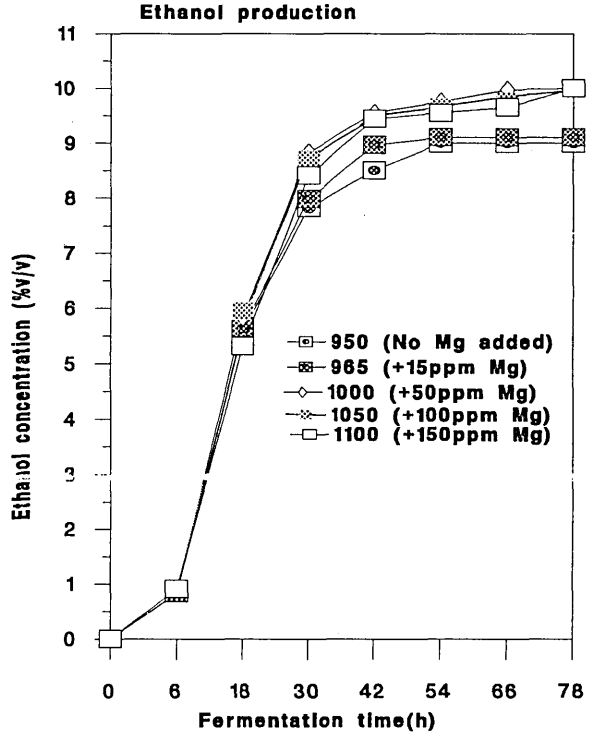
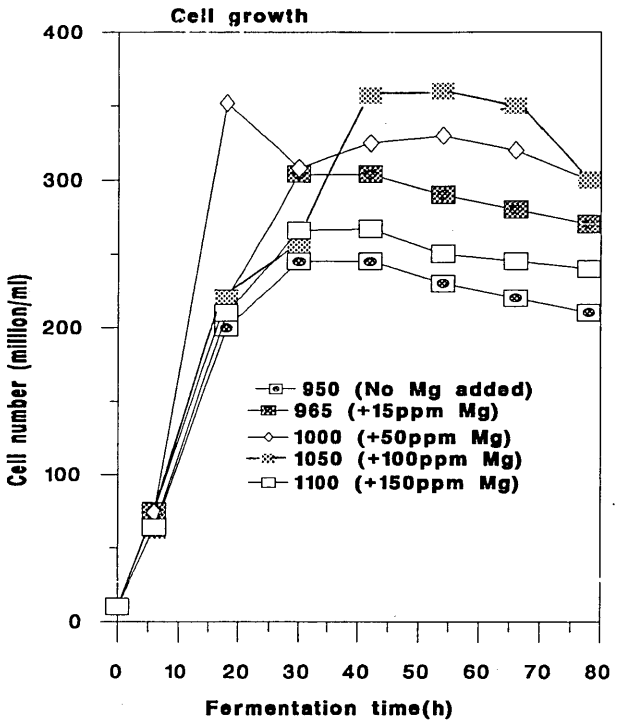
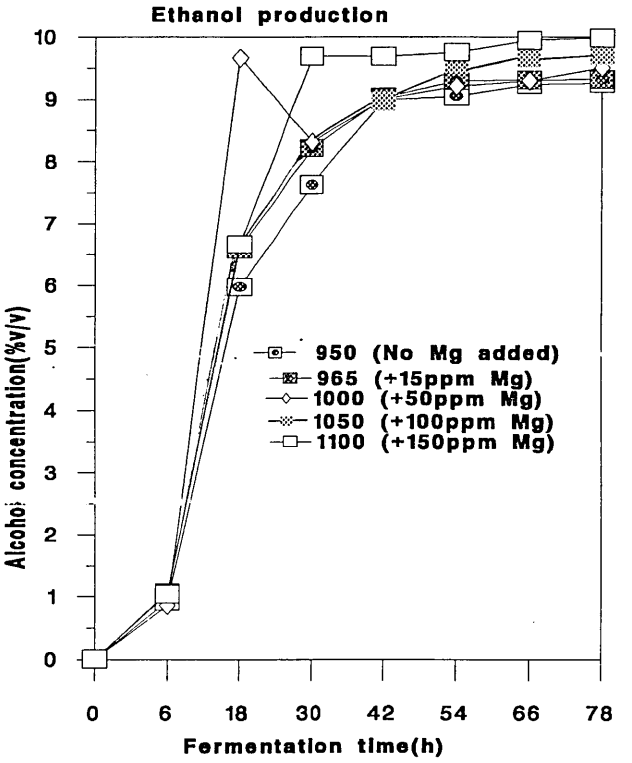


Fig 3.13 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (Saf-Levure baker's yeast) in Yeast extract Peptone Dextrose broth.

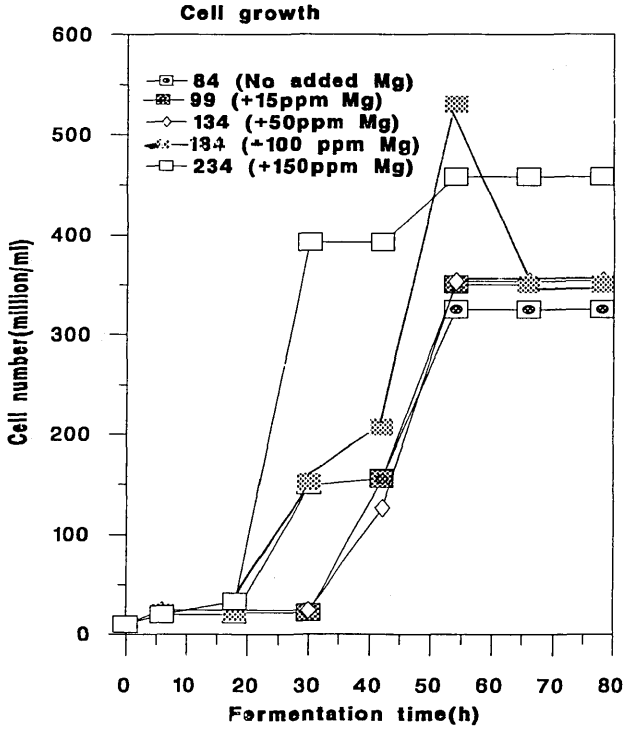




c) Malt wort fermentations

Results of fermentation experiments conducted in Mg^{2+}-supplemented malt wort using the DCL'M' distiller's yeast showed that all levels (15, 50, 100 and 150ppm) of Mg^{2+} were capable of stimulating cell growth and ethanol production (Table 3.3, Fig 3.14). The Red Star yeast in malt wort responded to added Mg^{2+} by rapid cell multiplication in the initial stage of fermentation. However, ethanol production at the later stages of fermentation was prolonged in response to higher levels (150ppm) of Mg^{2+} in the medium(Fig 3.15). The fermentation performances of Saf-Levure baker's yeast in Mg^{2+} -supplemented malt wort was similar to the other yeasts with positive effects observed towards cell growth and alcohol production (Table 3.3, Fig 3.16).

Fig 3.14 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in malt wort (1062 OG).



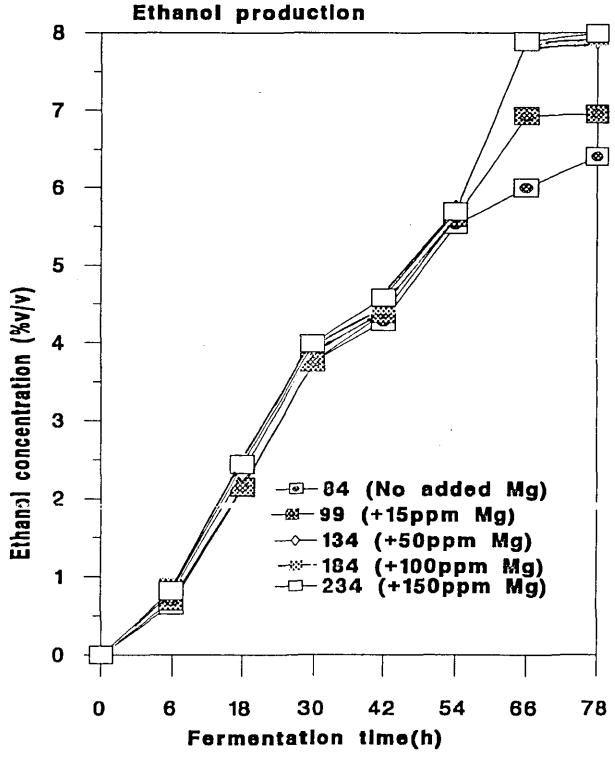
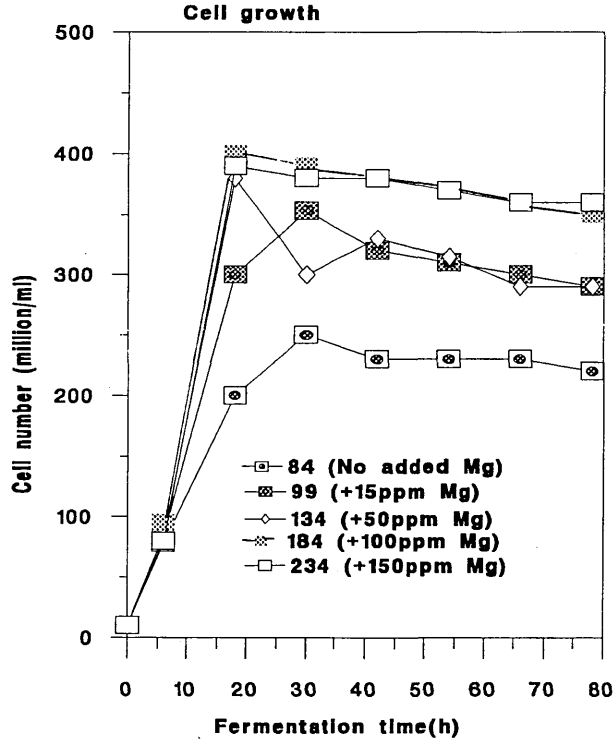


Fig 3.15 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (Red Star baker's yeast) in malt wort (1062 OG)



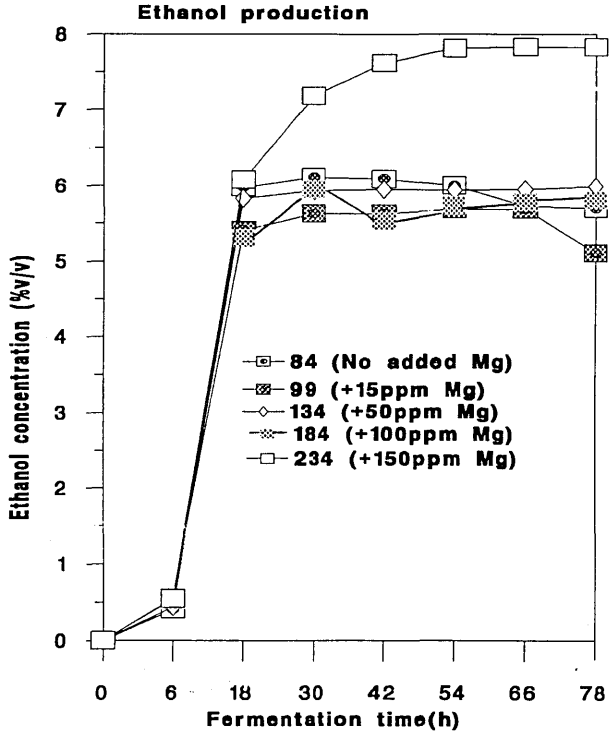
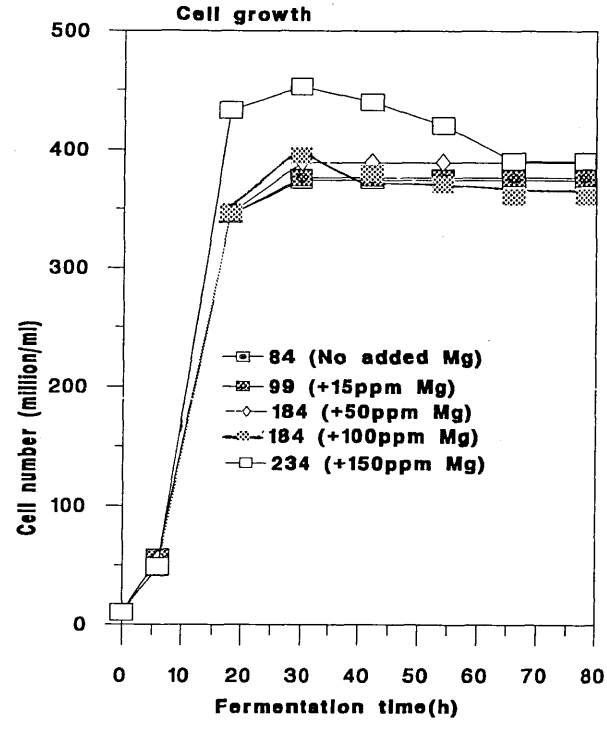
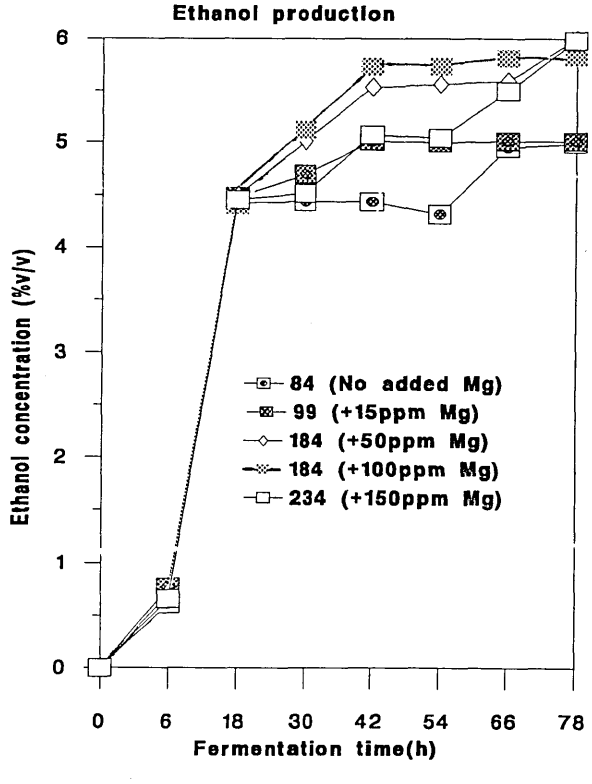


Fig 3.16 Effect of varying concentrations of Mg on cell growth and ethanol production by *Saccharomyces cerevisiae* (Saf-Levura baker's yeast) in malt wort(1062 OG).





Influence of Mg2+ on ethanol production and cell Mg content of DCL'M' yeast fermenting molasses

a) Ethanol production

Results of the experiments conducted to evaluate ethanol production by DCL'M' yeast in 10, 20, 25 and 30 % (w/v) concentrations of different types of molasses supplemented with 100ppm Mg^{2+} are summarised in Table 3. 4 and Figs 3.17-3.23. The results show a general stimulation of ethanol production in all higher molasses concentrations.

b) Cell Mg^{2+} content

The cell analyses of the samples collected from the above (30%w/v) molasses experiments, were performed to study the changes in cell Mg^{2+} during fermentation and the results are shown in Figs 3.24-3.27. In order to further study the uptake pattern of Mg^{2+} during the first 24h of fermentation, experiment was conducted in Sri Lankan molasses by sampling hourly intervals and analysing for cell growth, cell Mg^{2+} content, ethanol and residual sugar. Results revealed that yeast cells take up more Mg^{2+} during the exponential phase and later releases it in to the medium (Figs 28-31).

3.4 DISCUSSION

Addition of macronutrients into industrial fermentation media is a common practice in all yeast based industries. The nutrient ions that have electrochemical functions in cell metabolism are considered as major nutrients and include cations sodium, potassium, and magnesium, and anions chloride, nitrate, bi-carbonate and phosphate (Bown, 1966). Among the cationic nutrients, magnesium plays a vital role in the yeast cell life cycle being involved in: cell division control (Walker and Duffus, 1979, 1980); cell structural integrity, cell size modulation (Walker and Duffus, 1980); as an enzyme cofactor (Jones and Greenfield, 1984) and in the formation of high energy $Mg(ATP)^{2-}$ complexes (Heaton, 1990). Therefore a series of experiments were designed to evaluate the effects of addition of Mg^{2+} in industrial yeast media: molasses, malt wort and in laboratory yeast media: YEPD. Results of the present study revealed that all types of molasses differed in their total magnesium levels as follows:

Media	**Total Mg^{2+}** (ppm)
South African molasses	3600
Brazilian	3500
Mexican	2500
Sri Lankan	1900
Pakistani	1900
Javan	1300
Danish Beet	100
YEPD	950
malt wort	84

These analyses showed the total, not necessarily bio-available, Mg^{2+} presence in those media studied. Several workers have previous reported that most industrial media, especially molasses and malt wort, contain most of the macronutrients required for yeast growth and fermentation in their constituents (Chen and Chen, 1985; MacWilliams, 1968). Nevertheless, most complex commercial media are thought to be rich in organic and inoganic nutrients, Bovallius and Zacharias (1971) showed that there were variations in the metal ion contents , especially Mg^{2+}.

According to the findings of the initial experiments, adding 100ppm Mg^{2+} to South African molasses induced ethanol production in all yeast strains studied. Although the individual molasses wort had 3600ppm total Mg^{2+} as a constituent, external supplementation considerably influenced fermentation. Variation of ethanol production between the yeast strains studied could primarily be due to the fermentation capability of the yeast strain and to the intrinsic ethanol tolerance of the yeast. In unsupplemented South African molasses, ethanol production by yeast was according to following order:

L 2056>DCL'M'>L 2226> Baker's yeast> *S.pombe*> NCYC 1109> NCYC 679> *K.marxianus* (Table 3.1).

However, the response to added Mg^{2+} in comparison with the controls differed from the above order and as follows:

S.pombe>L2056>Baker's yeast>*K.marxianus* > DCL'M' > L2226>NCYC 679>NCYC 1109 (Table 3.1).

Similarly, YEPD broth (being a nurtient rich medium) contained about 950ppm Mg^{2+} and addition of an extra 100ppm Mg^{2+} caused an increase in ethanol production of the yeast strains. Ethanol production varied according to the following order:

L2226>L2056>NCYC1109>DCLBaker'syeast>DCL'M'>*S.pombe*>NCYC 679>*K.marxianus.*

Response to added Mg^{2+} in comparison with the controls were: L2226>NCYC 1109>>L 2056>Bakers yeast>*S.pombe*>*K.marxianus*>DCL'M'=NCYC 679 (Table 3.2).

Gray (1941) evaluated the fermentation capability of a number of yeast species for their ability to utilise glucose under various concentrations and found that variability of ethanol production capability and tolerance were species and strain dependent. According to Ismail and Ali (1971), the ethanol tolerance of *Saccharomyces cerevisiae* is under polygenic control and different strains of this species differ in their ability to both tolerateand produce ethanol. The response of yeast cells to added magnesium shows either the unavailability of the ion or ionic deficiencies (inadequate supply for the continuation of fermentation) in the media studied. The unavailability could be due to chelation, sequestration, adhesion or competition between other similar ions (Walker *et al*, 1990). The idea is supported by the findings of Pandy and Agarwal (1993) who showed the response of yeast to magnesium when existing cations were bound with a chelating agent (EDTA). Further, higher levels of ethanol produced by the ethanol intolerant yeast, *K.marxianus,* in YEPD broth clearly indicated the influence of media composition on fermentative capability of this yeast. These results compared with those of Casey and Ingledew (1986) who have emphasised the importance of environmental factors in dictating yeast ethanol tolerance.

When we consider the results of the experiments conducted to evaluate the performence of DCL'M', Red Star and Saf-Levure it is clear that all media lacked sufficient available Mg^{2+} to maximize yeast cell growth and fermentation. Closer observation of Figs 3.5-3.16 shows that in control fermentations (with no added Mg^{2+}) ethanol production as well as cell growth decreased sooner than the Mg^{2+} -supplemented flasks suggesting an

inadequate supply of this essential cation. Several workers have shown the importance of Mg^{2+} in yeast fermentation, cell division and cell size modulation (Walker and Duffus, 1980). However, variabilities are apparent between the strains and the growth media. For example, although Red Star and DCL'M' yeasts produced similar ethanol yields in control fermentations with no added Mg^{2+} in Sri Lankan molasses, in Mg^{2+} -supplemented media DCL'M' yeast was the highest producer. In South African molasses too, DCL'M' was the highest producer in controls as well as in Mg^{2+} -supplemented media. In malt wort, DCL'M' produced highest ethanol in the control and in Mg^{2+}-supplemented fermentations, whilst Red Star and Saf-Levure produced lower levels of ethanol. In YEPD broth, DCL'M' was the poorest fermenting yeast in controls and Red Star was the highest ethanol producer in the Mg^{2+} -supplemented YEPD media. These results show the suitability of DCL'M' and Red Star yeasts for molasses fermentations. However, unlike the strain DCL'M', Red Star is not a selected yeast for potable ethanol production and therefore other fermentation qualities such as the amount of by products other than ethanol should be taken into account before considering its use in distillery ethanol production. These interesting findings relate to the stimulatory effect of Mg^{2+} on fermentation of Sri Lankan and South African molasses by DCL'M' and Red Star yeasts again reveal the important influence of media composition and strain differences on fermentation.

The above experiments conducted in high gravity molasses media with Mg^{2+} supplementation using DCL'M' and baker's yeast (Table 3.3) showed that the distiller's yeast produced higher ethanol levels in magnesium enriched (additional 100ppm) media. However, baker's yeast, naturally a poor alcohol producer with lower alcohol tolerance, produced an appreciable amount of ethanol in magnesium -supplemented media compared to the control. This again demonstrated the beneficial influence of magnesium ions on ethanol production. In addition to stimulating fermentative activity in yeast, Mg^{2+} could also possibly exert a protective effect on cells exposed to adverse conditions created by high sugar or alcohol concentrations. The

above results are supported by the evidence that magnesium helps yeast cells to withstand adverse conditions created by ethanol and high osmotic pressure (Dombek and Ingram, 1986). Dasari *et al* (1990) and Walker *et al* (1994) suggested that magnesium may act to stabilise plasma membrane structure and to protect cells from environmental stress, including ethanol concentrations. However, the actual mechanism of the protective effect of magnesium remains to be elucidated and it could be due to the influence of magnesium ions on the activation of the genes responsible for the synthesis of heat shock proteins. However, further studies are necessary to investigate the influence of magnesium ions on the activation of heat shock genes in yeast. The response of yeast strains to added levels of Mg^{2+} could also be due to the changes in the cationic ratios in those fermentation media allowing higher chances for the added Mg^{2+} to bind with binding sites and thereby dominate the competition. The idea of the existance of competition between cations runs back to the work of Robertson (1958) who reported such a phenomenon between potassium and calcium. Legget *et al* (1965) reported the possibility of a competition between Ca^{2+}/Mg^{2+} and Fe^{2+}/Mn^{2+}. Ostero and Reyes (1994) reported that the K^{+}/Mg^{2+} ratio in molasses influenced growth and multiplication of the yeast *Candida utilis*. The reasons for the variable response of the same yeast in different molasses media therefore could be due to the actual competition between major cations as such media contain variable ionic compositions. Therefore, it is necessary to investigate the actual existance of such competition among the major cations in industrial media in order to establish the best cation combinations to achieve maximum alcohol production. Aspects of ionic interactions in yeast fermentations are considered in Chapter 4 of this thesis.

When we consider the experimental results of the fermentation experiments conducted to evaluate the performance of DCL'M' yeast in several types of molasses at different concentrations (10, 20, 25, 30w/v%), it was apparent firstly that these molasses solutions contained appreciable quantities of Mg^{2+} as their constituents. Ethanol productions in Mg^{2+} unsupplemented experiments were considered as controls and those results are

presented in Figs 3.17-3.23. Mg^{2+} addition enhanced ethanol yields irrespective of the type of molasses thus supporting the concept of inadequate Mg^{2+} bioavailability for the fermentation process due to chelating, sequestering and adsorbing compounds in molasses. Ethanol yields in different molasses may also vary due to the differing levels of organic and inorganic nutrients together with other compounds which may influence yeast fermentative metabolism. In Sri Lankan molasses, final ethanol yields were higher in Mg^{2+}-supplemented samples according to increasing concentration of molasses (Figs 3.17). The fermentation performance of yeasts in Mg^{2+} -supplemented South African molasses is shown in Table 3.4. According to the results the general tendency of enhanced ethanol production was similar to that of the Sri Lankan molasses although the final ethanol concentrations in 20%(w/v) molasses solutions did not differ between Mg^{2+} -treated and untreated media (Fig 3.18). Adding Mg^{2+} at all levels to Pakistani molasses showed stimulatory effects of Mg^{2+} toward fermentation (Fig 3.19). The response of DCL'M' yeast to Mg^{2+} in Javan molasses is shown in Fig 3.20. At 10%(w/v) molasses concentration, Mg^{2+}-treated cultures showed slight increases in ethanol production during the initial stages of fermentation. In 20%(w/v) molasses, there was no response to added Mg^{2+} during the exponential phase of fermentation, although there was a stimulatory effect towards the end of fermentation. However, in 30%(w/v) molasses, the influence of Mg^{2+} on ethanol production was significant even from the early stage of fermentation. Fermentation performance of the DCL'M' yeast in Brazilian molasses showed a higher response to added Mg^{2+} by producing more ethanol than the controls at all concentrations (Fig 3.21). Studies on Mexican molasses revealed that at lower concentrations (10%w/v) there was a poor response to added Mg^{2+}. Although the initial response to added Mg^{2+} in 20%(w/v) molasses was not prominant, there was a higher level of ethanol produced towards the end of fermentation. In 25%(w/v) molasses there was no positive influence of Mg^{2+} at the early stage of fermentation. In 30%(w/v) molasses yeast cells responded to added Mg^{2+} by producing higher ethanol levels throughout the fermentation (Fig 3.22). When beet molasses was supplemented with Mg^{2+} DCL'M' yeast produced higher levels of ethanol at all Mg^{2+}

Fig 3.17 Effect of Mg (100ppm) on ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in different concentrations (10, 20, 25, 30%w/v) of Sri Lankan molasses.

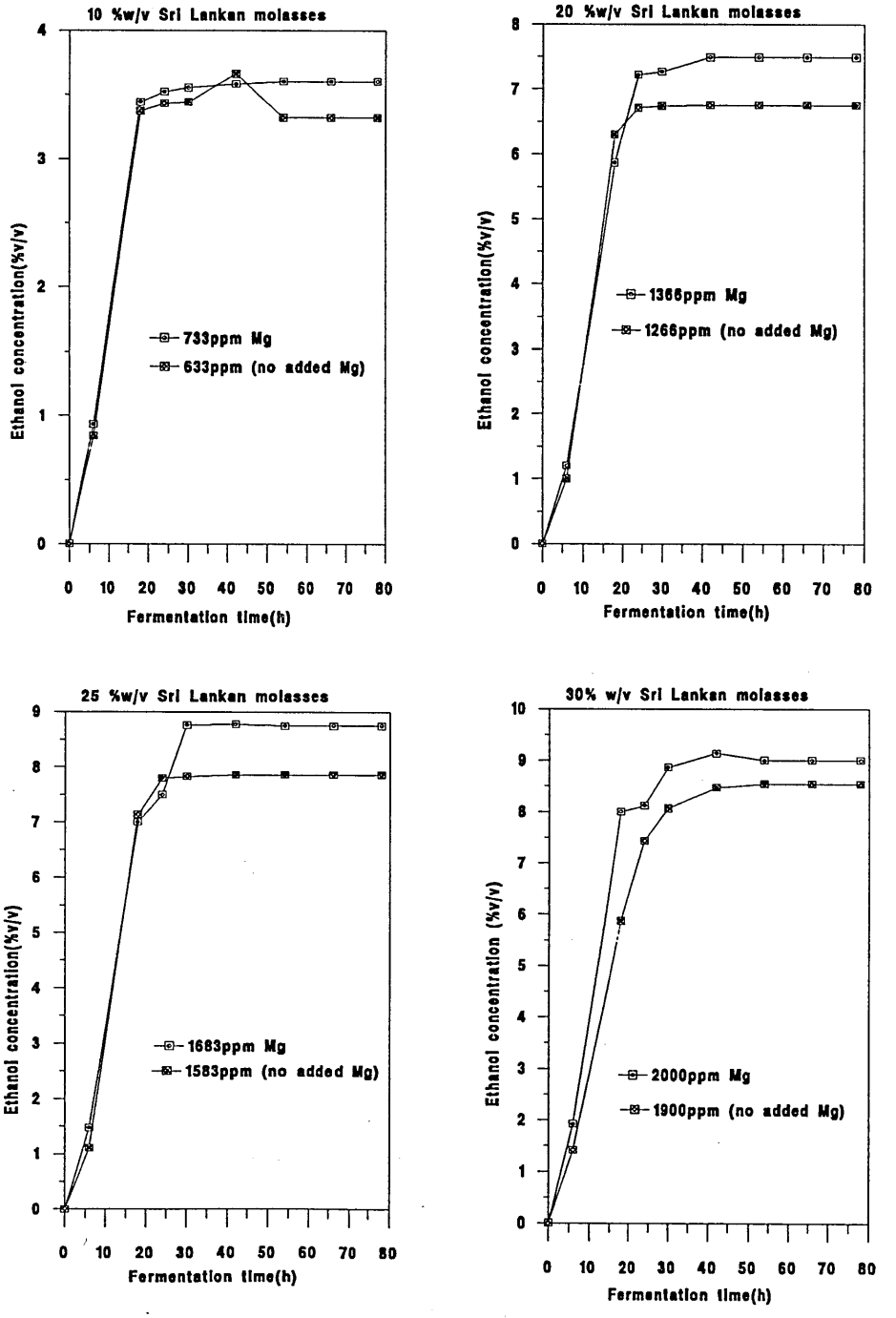


Fig 3.18 Effect of Mg (100ppm)on ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in different concentrations (10, 20, 25, 30%w/v) of South African molasses.

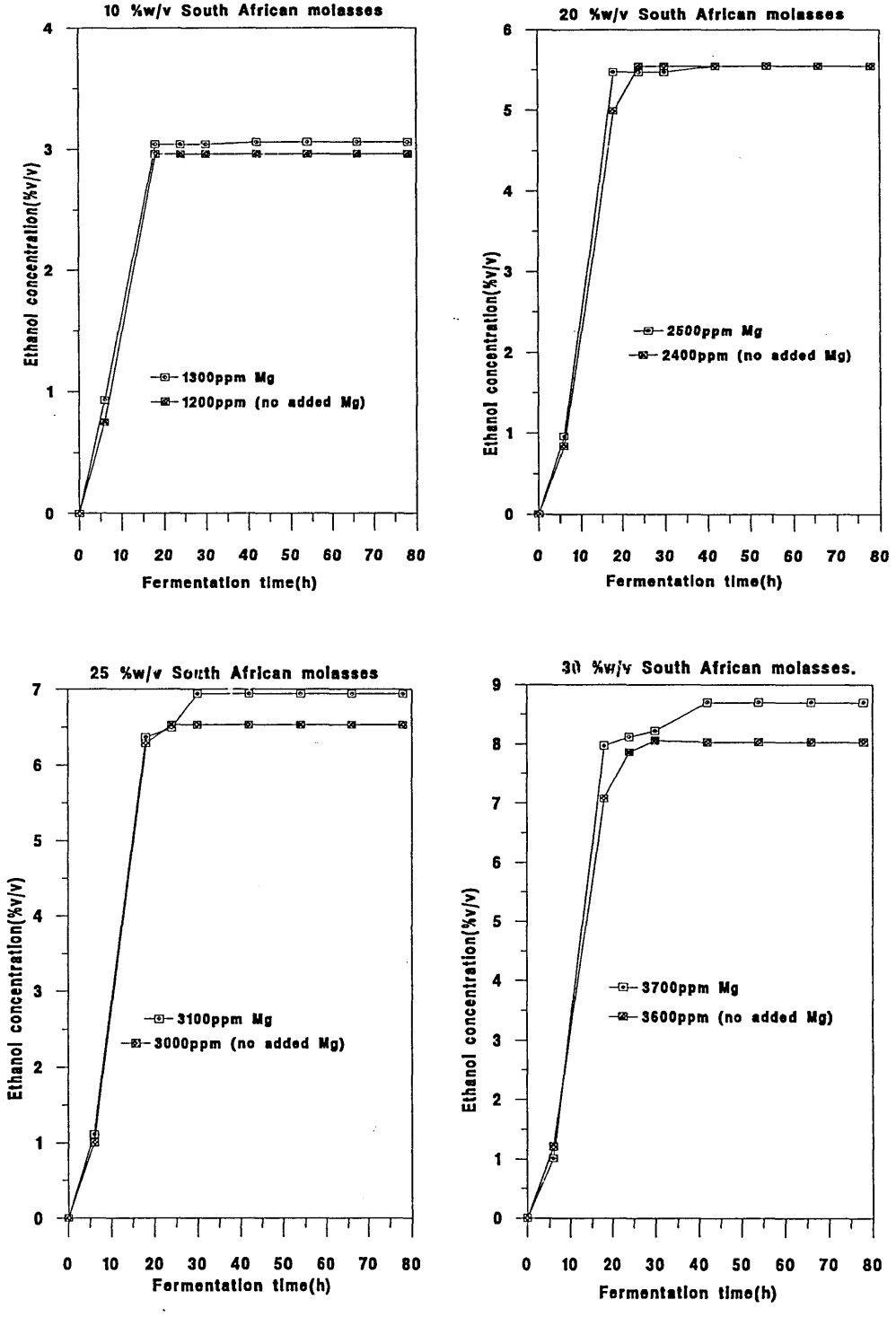


Fig 3.19 Effect of Mg (100ppm) on ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in different concentrations (10, 20, 25, 30%w/v) of Pakistani molasses.

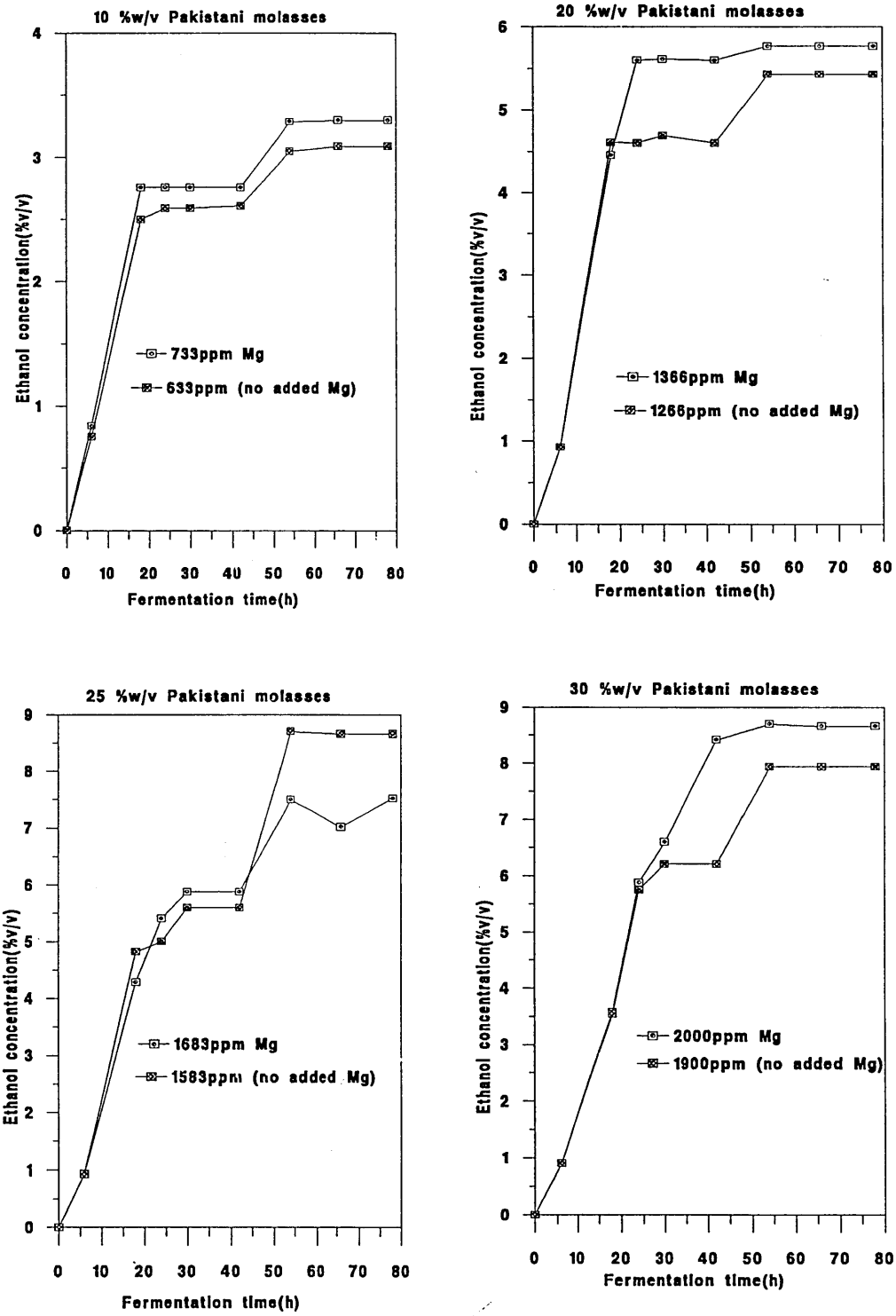
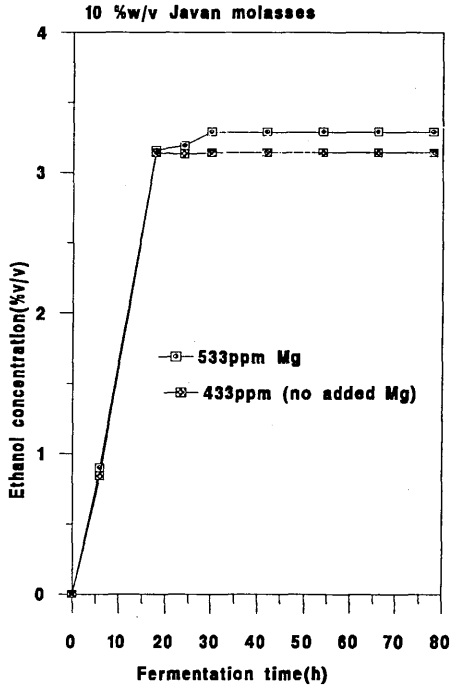
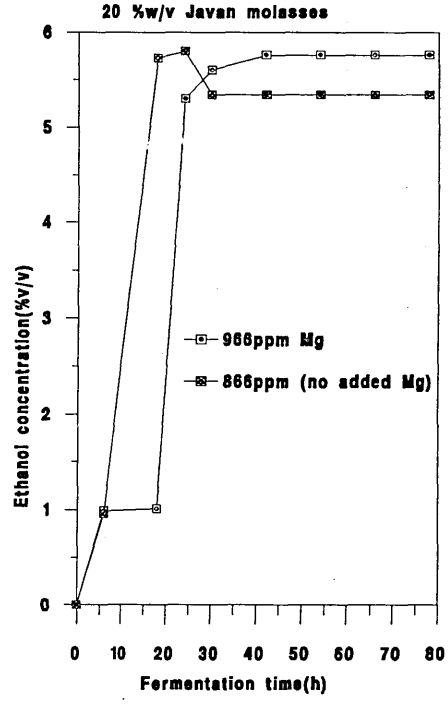
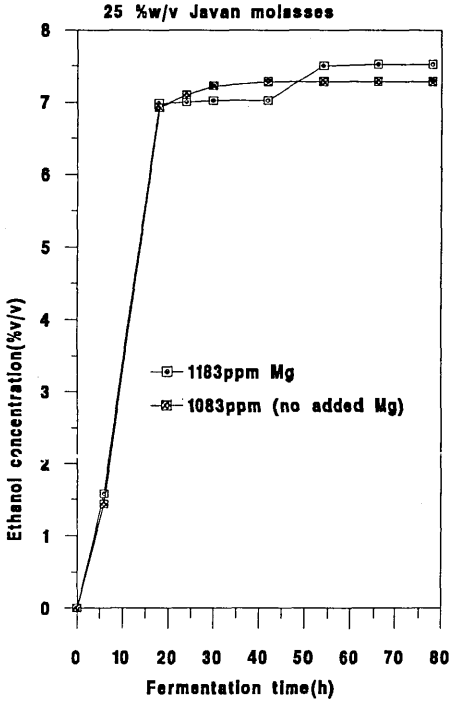


Fig 3.20 Effect of Mg (100ppm) on ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in different concentrations (10, 20, 25, 30%w/v) of Javan molasses.







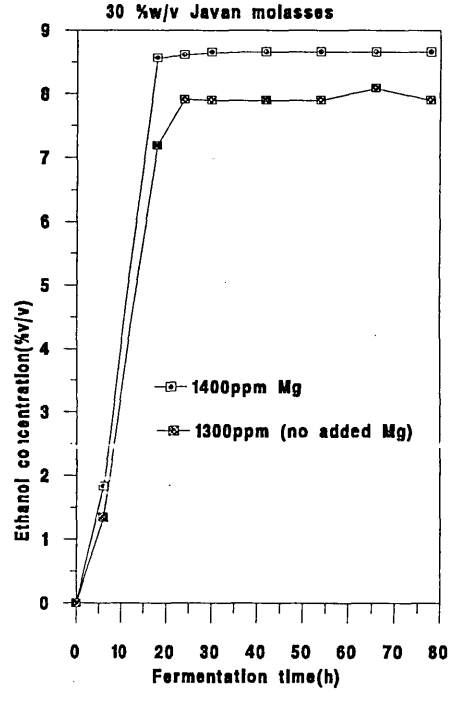


Fig 3.21 Effect of Mg (100ppm) on ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in different concentrations (10, 20, 25, 30%w/v) of Brazillian molasses.

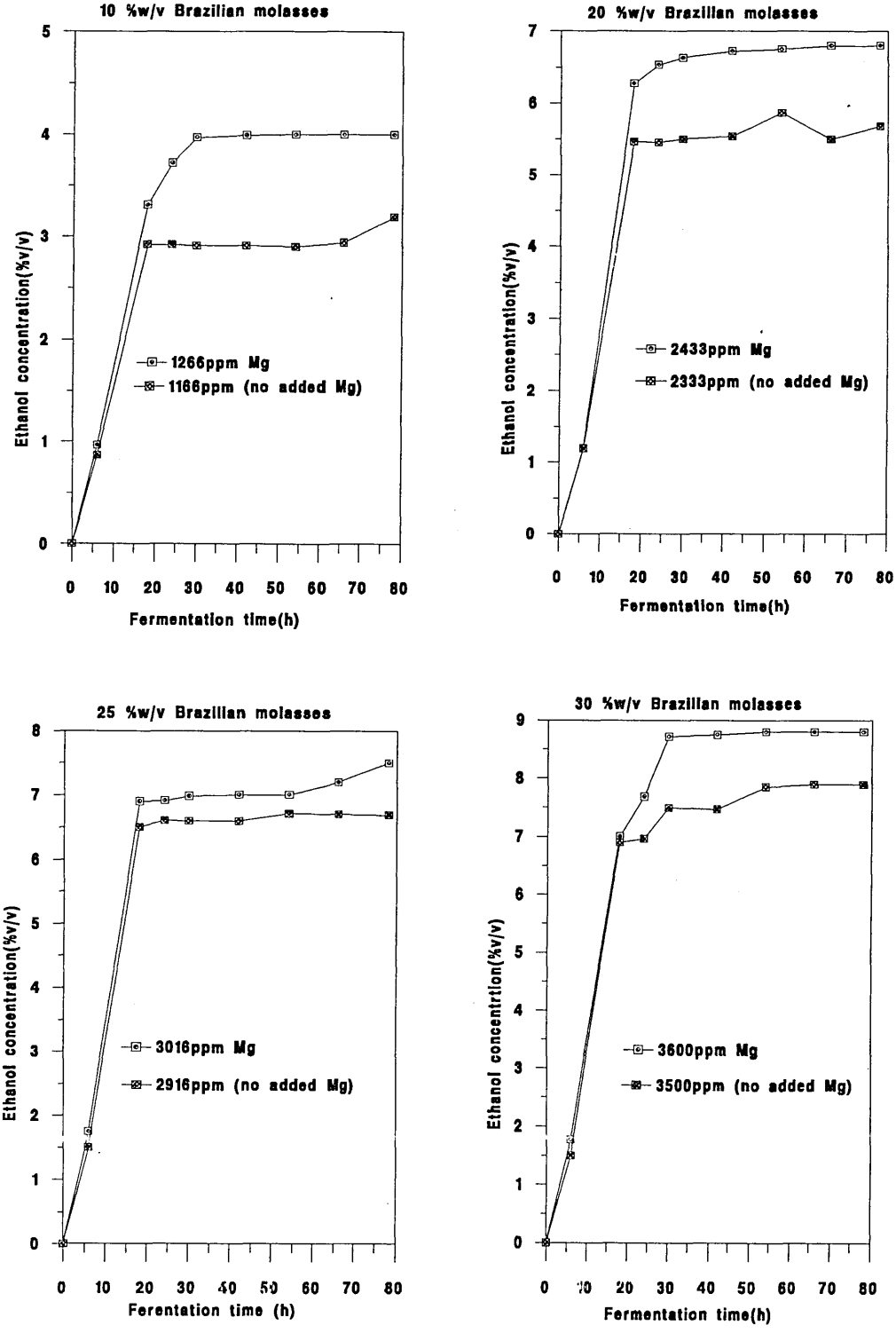


Fig 3.22 Effect of Mg (100ppm) on ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in different concentrations (10, 20, 25, 30%w/v) of Mexican molasses.

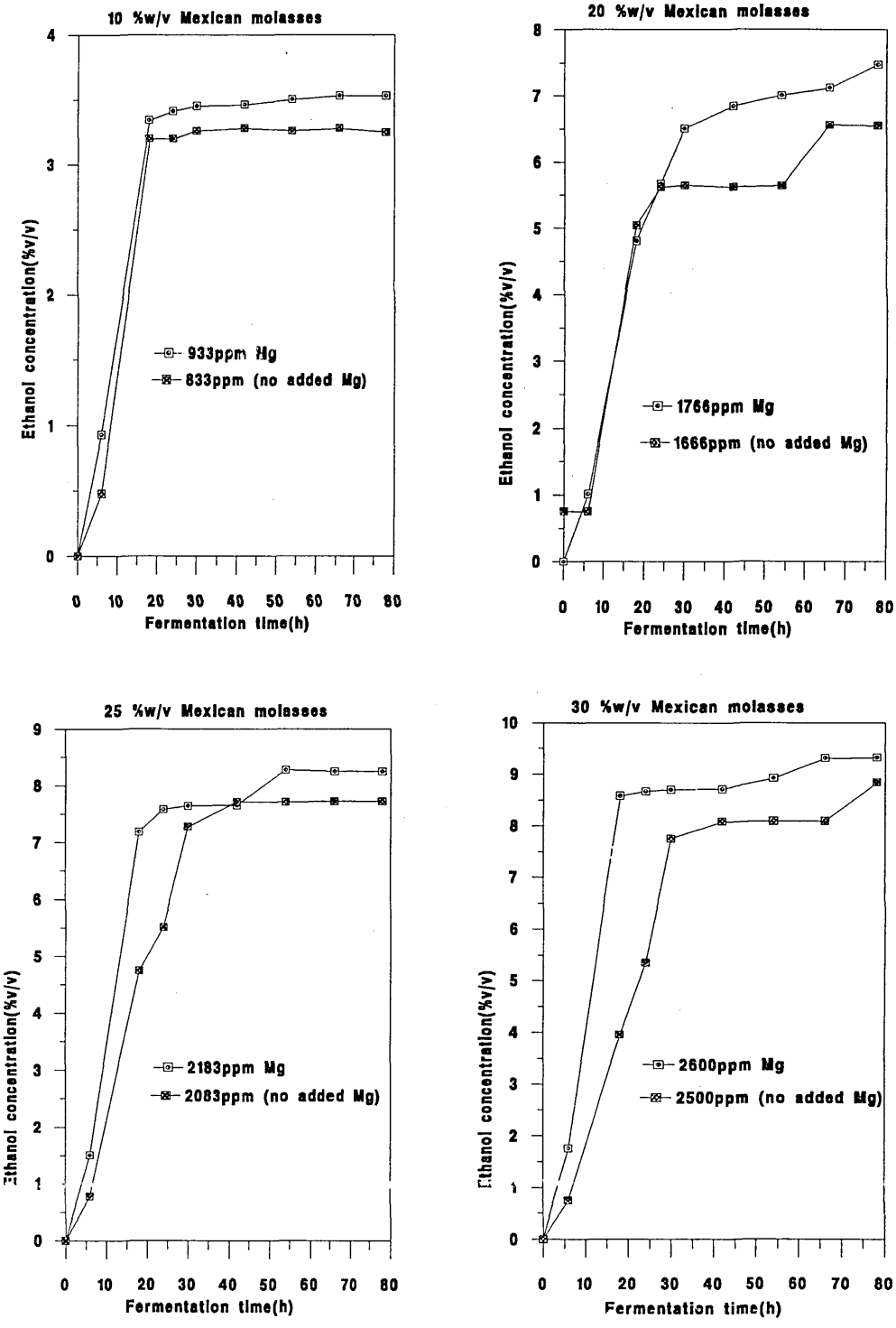
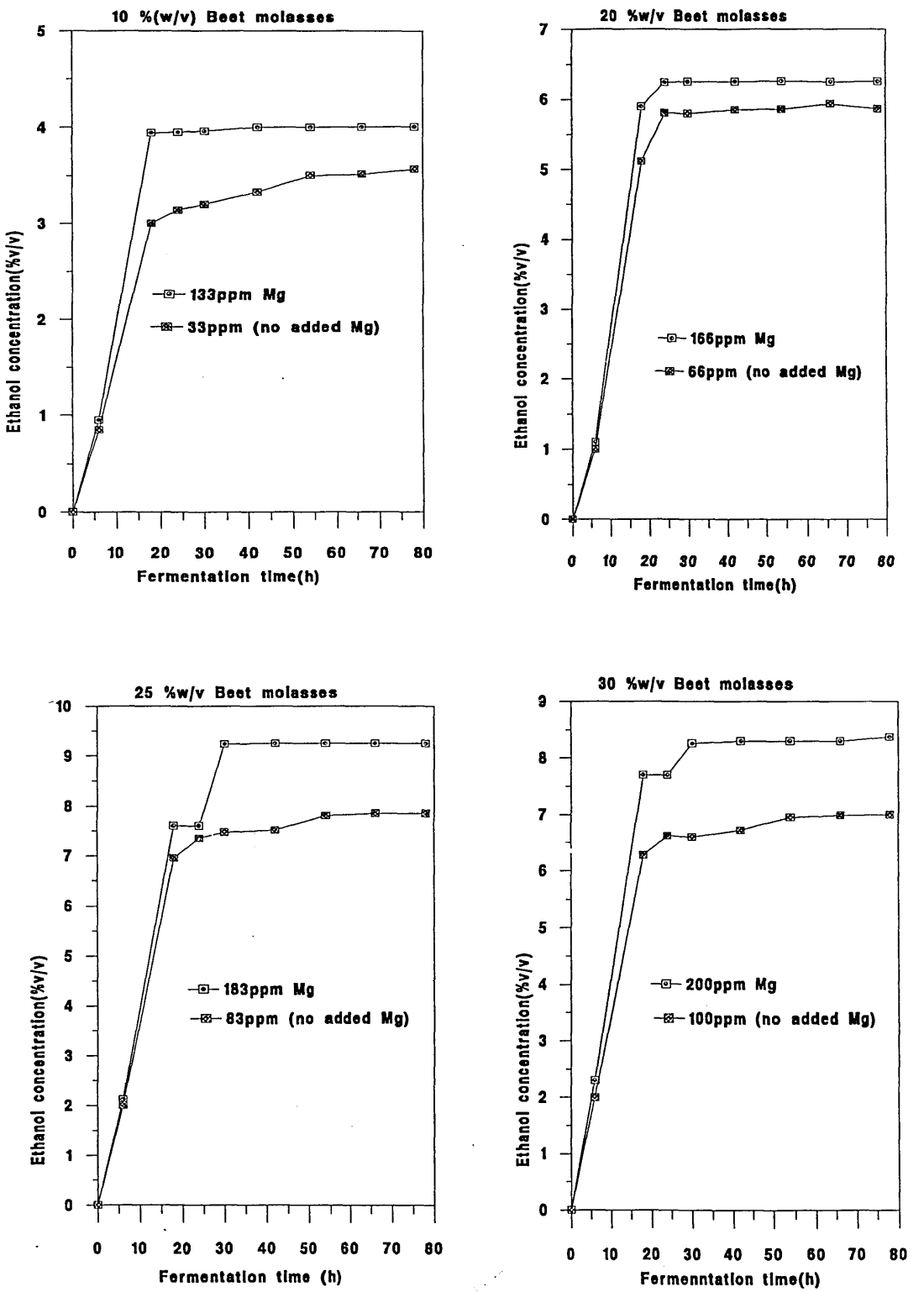


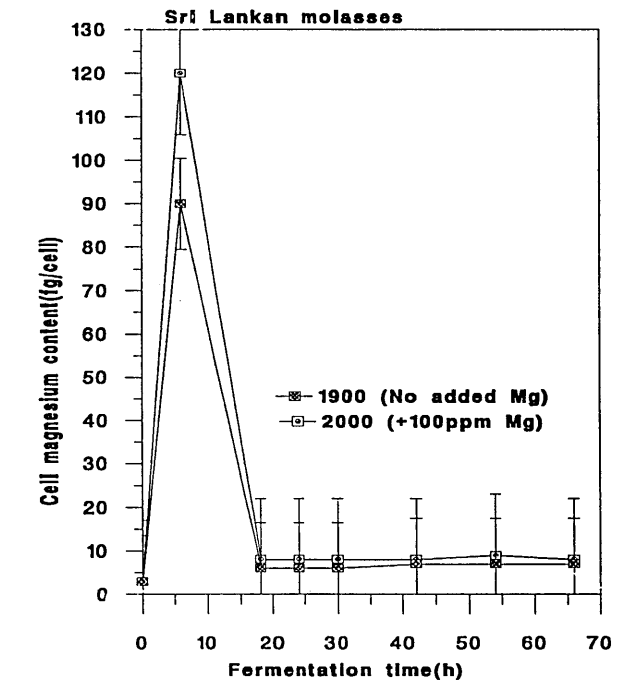
Fig 3.23 Effect of Mg (100ppm) on ethanol production by *Saccharomyces cerevisiae* (DCL'M' distiller's yeast) in different concentrations (10, 20, 25, 30%w/v) of Danish beet molasses.



concentrations (Fig 3.23). Present results are supported by the experimental findings of Wolniewiez *et al*, (1988) who showed a stimulatory response on ethanol production of added magnesium sulphate into molasses.

Samples collected from 30% (w/v) molasses fermentations were analysed for cellular Mg^{2+} in order to study patterns of Mg^{2+} uptake. The results are shown in Figs 3.24-3.27. Closer analysis of the above results revealed that peaks in Mg^{2+} absorption by cells coincided with the exponential growth phase of the fermentation. Therefore, it is clear that yeast cells uptake magnesium during their exponential phase with later release to the medium. Perhaps an influx and efflux perturbation mechanism exists for Mg^{2+}, as is known for other cations in yeast. However, most of these mechanisms employed by yeast are to remove toxic or unwanted metals from the cells. For example, Asensio *et al*, (1976 cited by Borst-Pauwels, 1981) reported that yeast cells efflux Li^{+} to maintain very low intracellular concentrations. Similar systems exist for Na^{+} and K^{+} efflux during divalent cation uptake by yeast cells. It is widely believed that a Ca^{2+} -dependent specific extrusion pump exists which controls the uptake of Mn^{2+}over Sr^{2+} in *S. cerevisiae* (Theuvenet *et al* 1976). Boutry *et al* (1977) have also reported the existence of a Ca^{2+} -efflux system in yeast cells. Yeast cells uptake mono and divalent cations together with $H_2PO_4^-$ ions and preferentially locate them in cell vacuoles. According to Lichko *et al* (1982), the ratio of total ions to cytoplasmic ions is of the order of 4 for K^{+} , 4-28 for Mg^{2+} , and 10 for Ca^{2+}. Such a localisation help cells to maintain the cytoplasmic concentration at low levels. However, the cells never efflux all the Mg^{2+} during their life cycle. This is clear in all cell analysis which suggests that although yeast cells follow Mg^{2+} influx and efflux systems they always maintain a minimum cellular Mg^{2+} level. Maynard (1993) suggested that this Mg^{2+} could be involved in the structural integrity of cells and the effluxed Mg^{2+} can be considered as "enzymatic" Mg^{2+} which is required during peak metabolic activities like fermentation.

Fig 3.24 Changes in cellular Mg of *Saccharomyces cerevisiae* (DCL'M' distillers yeast) during fermentation of Sri Lankan and South African molasses.



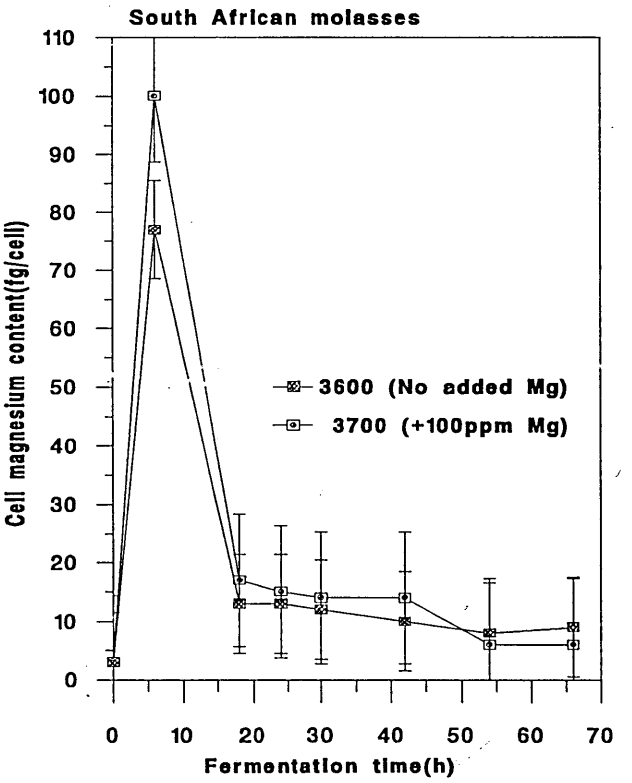
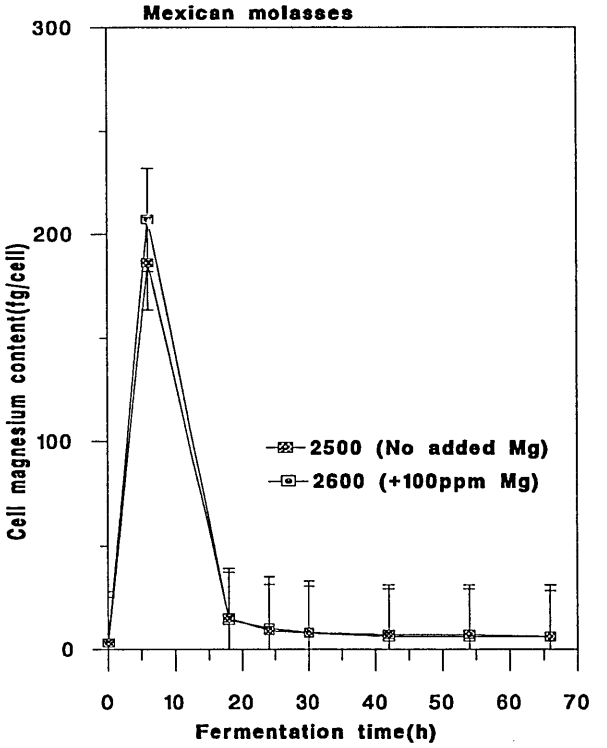


Fig 3.25 Changes in cellular Mg of *Saccharomyces cerevisiae* (DCL'M' distillers yeast) during fermentation of Mexican and Pakistani molasses.



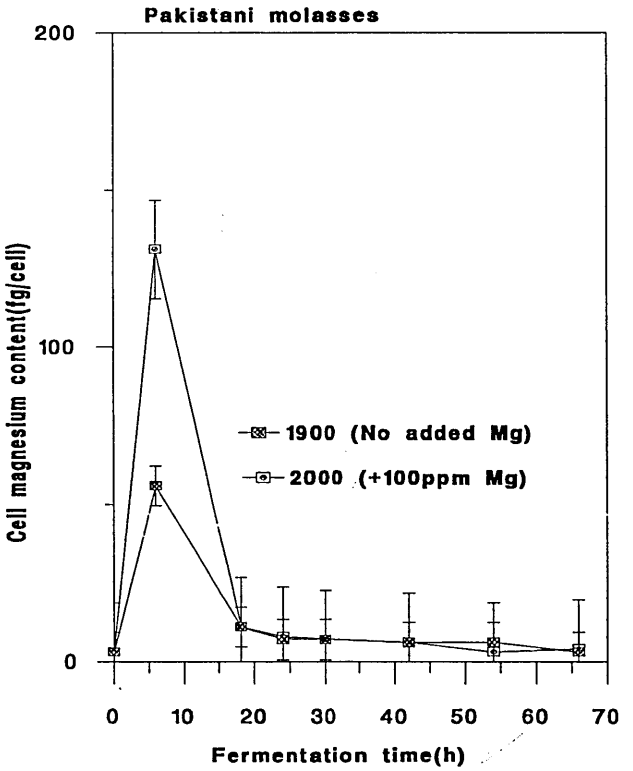
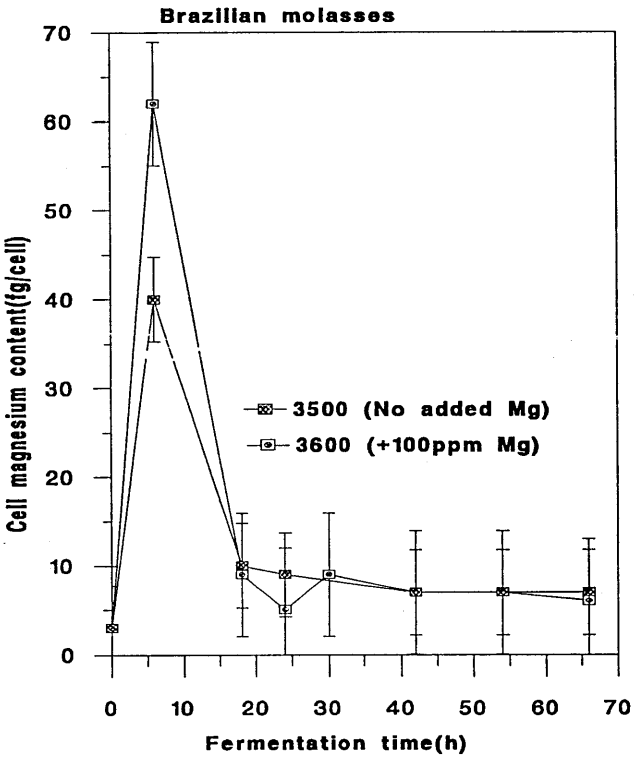


Fig 3.26 Changes in cellular Mg of *Saccharomyces cerevisiae* (DCL'M' distillers yeast) during fermentation of Brazillian and Javan molasses.



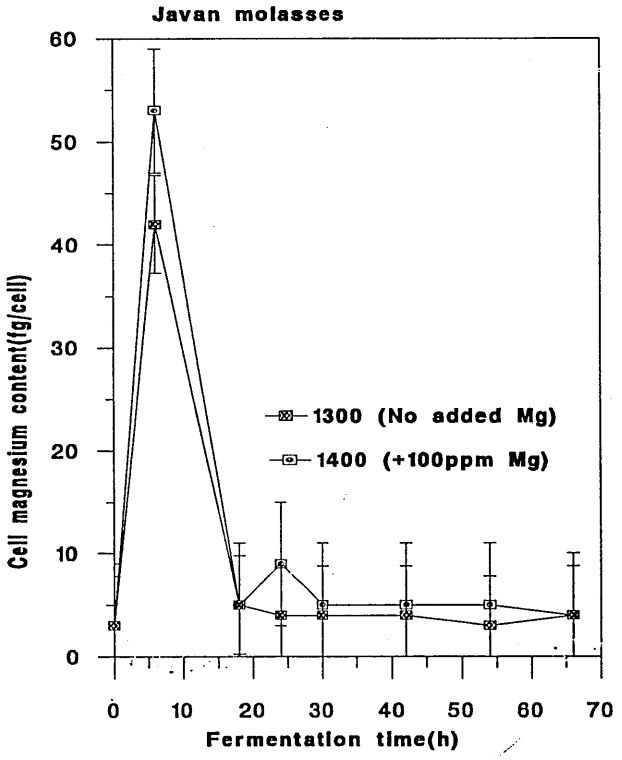
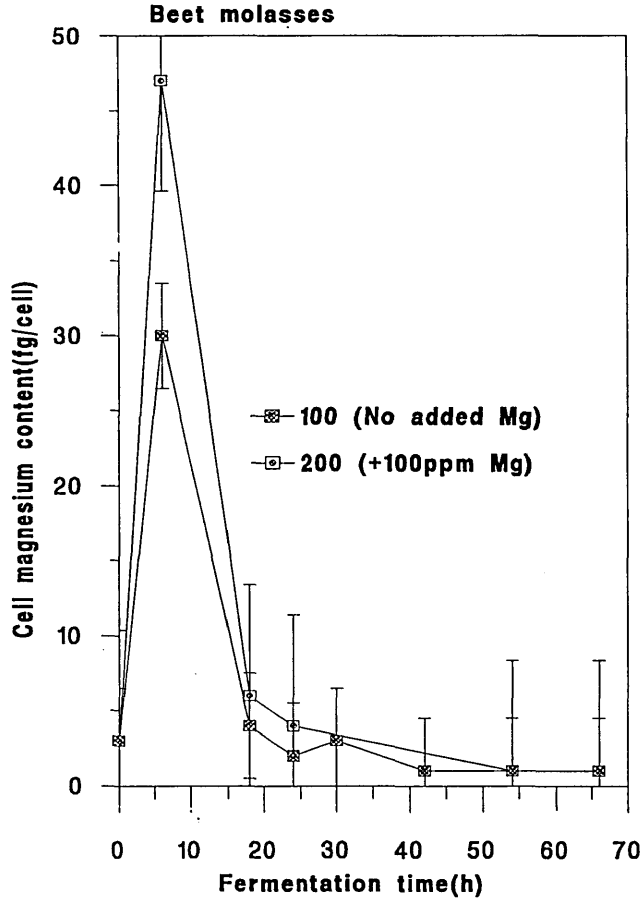


Fig 3.27 Changes in cellular Mg of *Saccharomyces cerevisiae* (DCL'M' distillers yeast) during fermentation of Danish beet molasses.



In order to further clarify this evidence, studies were conducted to investigate the magnesium uptake patterns of DCL'M' yeast during the early hours of fermentation in Mg^{2+} supplemented medium by hourly sampling and analyses for cell numbers, ethanol, residual sugars and cellular Mg^{2+}. The results revealed that the yeast cells uptake Mg^{2+} during exponential growth followed by later releases (Fig 3.28, 3.29). Further, the increasing uptake of Mg^{2+} coincided with increasing ethanol production (Fig 3.30). Residual sugar analysis showed a decreasing trend with increasing cell number and ethanol production in response to Mg^{2+}-supplementation (Fig 3.31). Maynard (1993) reported that magnesium ions transported by *S.cerevisiae* appear to correlate with fermentative activity. Therefore, it would be interesting to understand the behaviour of yeast cells if they were given necessary quantities of Mg^{2+} or if they were Mg^{2+}-preconditioned prior to fermentation in order to exploit these Mg^{2+} transport phenomenon.

Fig 3.28 Influence of Mg on cell growth of DCL'M' yeast in Sri Lankan molasses

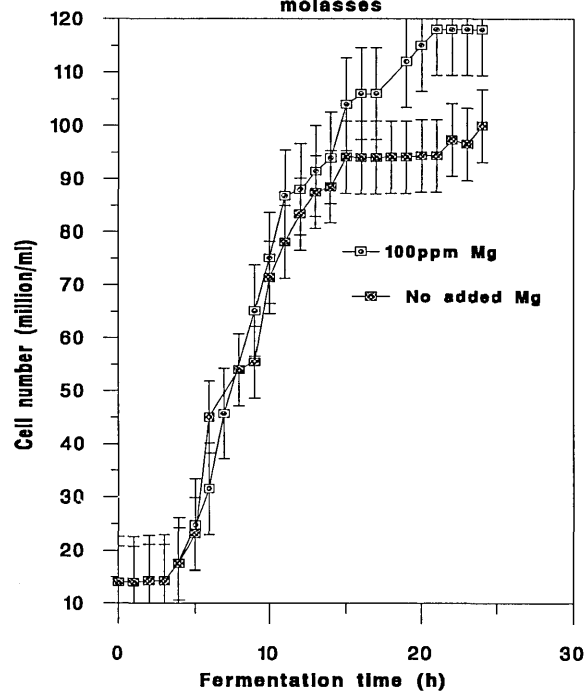


Fig 3.29 Uptake pattern of Mg by DCL'M' yeast during Sri Lankan molasses fermentation

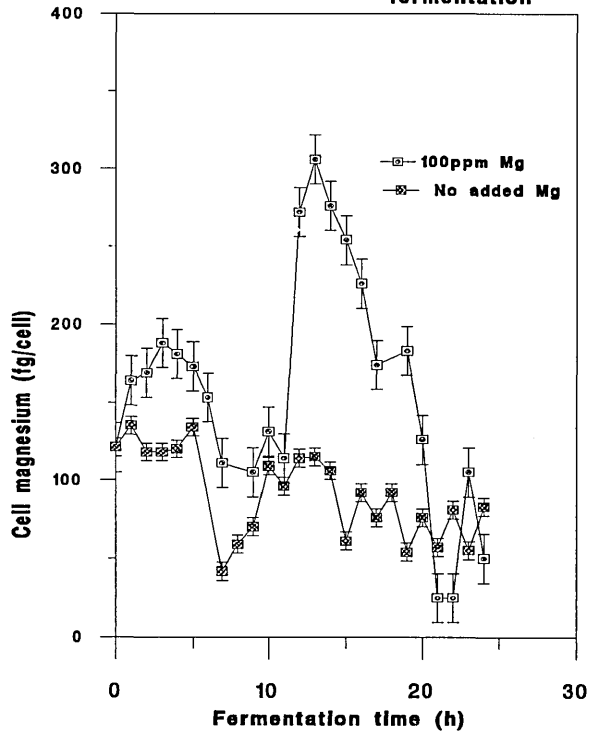


Fig 3.30 Influence of Mg on progress of ethanol production of DCL'M' yeast in Sri Lankan molasses

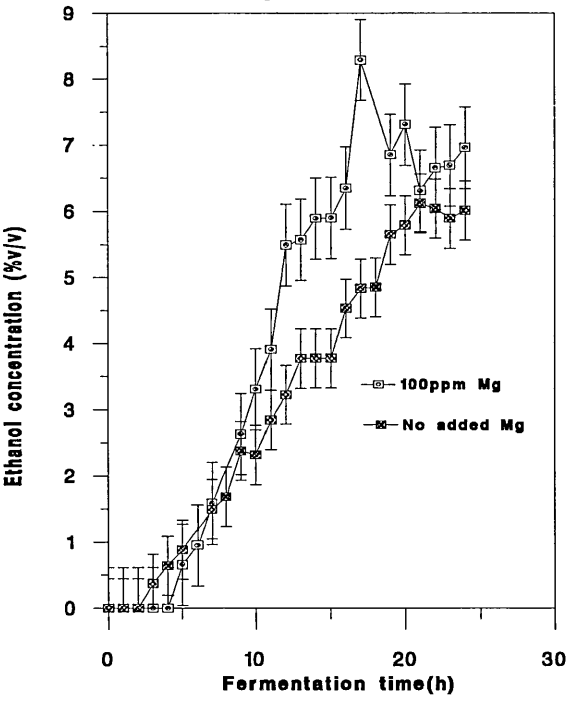
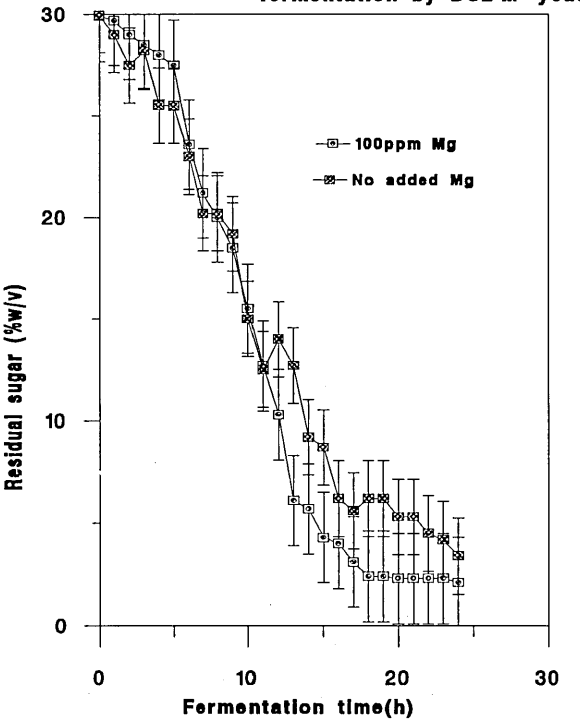


Fig 3.31 Influence of Mg on sugar consumption from Sri Lankan molasses during fermentation by DCL'M' yeast



CHAPTER 4: Interactions between K^+, Mg^{2+}, Ca^{2+}, and Zn^{2+} ions in yeast fermentations

4.1 Introduction

Among the cationic yeast nutrients, potassium, magnesium, calcium and zinc are involved in structural and enzymatic regulatory activities during growth and metabolism. Potassium is mainly involved in osmoregulation, charge-balancing and in regulation of divalent cation and phosphate uptake into the yeast cell (Jones and Greenfield, 1984). Magnesium is essential for yeast growth, metabolism and fermentation. It is also an essential cation in nucleic acid synthesis and as cofactor of more than 300 enzymes including hexokinases, phosphofructokinases, phosphoglycerate kinase, pyruvate kinase and enolase in glycolysis (Walker, 1994). Calcium requirements for yeast growth are debatable. The presence of Ca^{2+} in fermentation media may also compete with essential divalent cations like Mg^{2+} (Walker *et al*, 1996) and cause growth inhibition at high concentrations (Slatukoglu and Slaughter, 1983). Zinc is an essential micronutrient in yeast metabolism. It functions as a cofactor of essential enzymes such as alcohol and aldehyde dehydrogenases which are directily involved in ethanol production. Recent publications highlight the influence of this cation on wort sugar uptake and fermentation performance of brewers yeast (Desmartez *et al*, 1993). The bioavailability of these cations directly influence sugar metabolism by yeast in brewing and distillery fermentations and so the correct balance of these cations is vital for successful production of ethanol (Walker et al, 1996). Knowledge of interactions between K^+, Mg^{2+}, Ca^{2+} and Zn^{2+} , as well as their relative bioavailability, in industrial growth substrates is important in order to optimally manipulate their levels in fermentations.

This Chapter examined the significance of cationic (K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}) interactions in industrial media (molasses and malt wort) and estimated quadratic response surfaces to establish optimum combinations of the cations for maximial ethanol production by yeast.

4.2. Experimental Approach

Yeast culture and fermentation conditions

Saccharomyces cerevisiae distillers strain (DCL'M') was used for the fermentation studies. Stock cultures of yeast were maintained at 4°C on malt extract agar slopes and regularly subcultured by incubating slopes at 30° C for 1-2 days. Seed inocula for experimental fermentations were prepared from 1-2 day old cultures in the same liquid medium as for individual fermentations.

Synthetic media fermentations

Experimental fermentations using *S. cerevisiae* were conducted at 30°C in a rotary incubator with defined growth media which mimicked sugarcane molasses (21°Brix) and malt wort (OG 1040). Low , intermediate and high levels of K^{+}, Mg^{2+}, Ca^{2+} and Zn^{2+} in these media were then chosen following a literature survey of maximum and minimum concentrations (Baker, 1979; Boze et al, 1992; Lentini *et al* 1990; Chen and Chen, 1985; MacWilliams, 1968; Underkofler and Hickey, 1954). Table 4.1 shows the levels chosen for experimental fermentations.

Table 4.1 Cation Combinations (ppm) in Synthetic Media fermentations

• Molasses

Cation	Literature range in 21° Brix molasses		Average levels in experiments*		
	High	**Low**	**High**	**Int**	**Low**
Mg	277	9	270	70	16
Ca	2222	278	1027	509	71
K	9585	2955	7251	5102	2159

• Malt wort

Cation	Literature range in OG 1040 wort		Average levels in experiments*		
	High	**Low**	**High**	**Int**	**Low**
Zn	0.54	0.05	0.48	0.26	0.11
Mg	150	15	193	102	21
Ca	200	23	244	36	30

Int = intermediate

*These cation levels were chosen inorder to represent high, intermediate and low levels in molasses and malt wort.

The synthetic molasses medium comprised: sucrose (180g,) $(NH_4)_2SO_4$ (5 g), $(NH_4)_2PO_4$ (1.42g), NaCl (0.5g), $MgSO_4.7H_2O$ (variable), $CaCl_2.6H_2O$ (variable), KCl (variable), micronutrients: H_3BO_3 (0.5mg), $MnSO_4.H_2O$ (0.4mg), $ZnSO_4.7H_2O$ (0.4mg), $FeCl_3.6H_2O$ (0.2mg), $H_2MO_4.H_2O$ (0.16mg), KI (0.10mg), $CuSO_4.5H_2O$ (0.04mg) and citric acid (1.0mg), vitamins: inostol (10mg), nicotinic acid (10mg), calcium pantothenate (1mg), biotin (0.01mg), pyridoxine hydrochloride (0.04 mg), thiamine hyrochloride (0.04mg) para-aminobenzoic acid (2mg) , ergosterol 2ml and ultrapure water (18MΩ) to 1000 ml.

The syntetic malt wort comprised: glucose (10g), fructose (3.3g), sucrose (5.3g), maltose (38.9g), maltotriose (1.3g), maltotetraose (5.3) dextrin (24.8g), $(NH_4)_2SO_4$(5g), $(NH_4)_2$ PO_4 (1.42g), NaCl (0.5g), KCl (1g), $MgSO_4.7H_2O$ (variable) and $CaCl_2$ (variable). Micronutrients were as in synthetic molasses, except that zinc source ($ZnCl_2$) was variable. Vitamins and ergosterol were supplemented as in the synthetic molasses wort and ultrapure water to 1000ml.

Growth media for the seed inoculum comprised: glucose (50g), KCl (1g), $CaCl_2.6H_2O$(10mg), $MgSO_4.7H_2O$ (0.14g) in synthetic molasses and similar contents of K, Mg, Ca together with $ZnSO_4.7H_2O$ (0.20mg) for the malt wort. The contents were dissolved in 1000 ml of ultrapure water. Seed inocula were prepared as follows. Actively growing yeast cells on malt extract agar slopes were harvested and inoculated into 200 ml of growth media for seed inoculum and incubated for 48h at 30°C in a rotary incubator with 150 r.p.m agitation. After incubation cells were separated by centrifugation and washed 4 times with ultrapure water and used for inoculation.

Experimental design and synthetic media fermentations

Two "3^3" experiments in molasses and malt wort were designed with three-factor factorial with 3 cations each at three levels (low , intermediate and high - see Table 4.1). Erlenmeyer flasks (250 ml) were deionised by soaking in 2% v/v nitric acid solution overnight and washing in ultrapure water followed by a rinse with 0.1M EDTA to remove any nitrate traces and a final wash (three times in ultrapure water) to completion. Initially, sterilized synthetic media was added to flasks followed by sterilized salt solutions (KCl, $MgSO_4$, $CaCl_2$ for synthetic molasses and $MgSO_4$, $CaCl_2$, and $ZnCl_2$ for synthetic malt wort) were added to achieve the cation combinations as in Table 4.1. Flasks were then inoculated with washed yeast inocula grown as previously described. The initial cell densities were approximately 5×10^6 cells/ml. Fermentations were carried out at 30°C in a rotary incubator with 75 r.p.m. agitation to keep yeast cells in suspension. All fermentations were duplicated.

Authentic molasses and malt wort fermentations

Fermentations were conducted in several geographic sources of molasses (21° Brix, cane and beet) and two kinds of ale or lager malt wort (1040 S.G, Scottish and Newcastle brewery and laboratory extracted wort). Laboratory extraction was done by infusion mashing of 60g of Prisma variety malt grist in a flask containing 250 ml of ultrapure water for 70 min at 68°C in a water bath maintained at 71°C. The cooled mash was then centrifuged to separate the wort. Weight of the inital grist and the S.G. of the final wort was adjusted as required. Cation concentrations of the media were determined prior to fermentations as described below. Ratios of Mg^{2+} and Zn^{2+} in the malt worts were altered by adding 50 ppm Mg^{2+} and 0.26 ppm Zn^{2+} into laboratory extracted wort and similar amounts of Mg^{2+} and 0.20 ppm Zn^{2+} into brewery wort prior to fermentations (Table 4.2).

Table 4.2 Cation combinations for authentic media fermentations

Molasses	**Cations(ppm) K/Ca/Mg**	**Malt wort**	**Cations(ppm) Zn/Ca/Mg**
Sri-Lankan	2000/400/160	Lab. extract	0.18/55/84
Brazilian	1100/600/210	Lab.ext.(alt)	0.34/55/134
Javan	1500/200/50	Brewery	0.24/60/105
Danish(Beet)	2200/230/23	Brewery (alt)	0.44/60/155
Mexican	1600/600/60		
Pakistani	2500/600/160		
S. African	3500/600/200		

alt = altered

Measurement of fermentation parameters

Ethanol, cell growth and biomass

Microcentrifuged fermented wort samples were analysed using gas chromatography (Hewllet Packard 5710A) with isopropanol (5%,v/v) as an internal standard. Yeast growth was determined using a Coulter Multisizer (Coulter Electronics Ltd., Luton, UK) following appropriate dilutions of culture samples in Isoton II containg 0.9% (w/v) NaCl. Cell biomass of the samples collected from each treatments were determined as described in the Methods section (2.2.6).

Metal ion analysis

Total Mg^{2+}, Ca^{2+}, and Zn^{2+} concentrations in yeast cells and fermentation media were analysed using atomic absorption spectrophotometry (Perkin-Elmer 1100B) with external standardization. K^{+} ions were determined by flame photometry (Corning model 400). For yeast growth media annlysis, 10 ml samples (unfermented and fermented) were centrifuged and supernatants appropriately diluted in deionized, ultra pure water. For cellular metal ion contents, aliquots (1 ml) from thoroughly mixed fermentors were microcentrifuged, cell pellets washed 4 times in ultrapure water, resuspended in 1 ml of ultrapure water and finally digested in 1 ml concentrated H_2SO_4 at 100°C for 30 min. The cellular hydrolysates were further diluted in ultra pure water prior to atomic absorption spectrophotometry and flame photometry.

Data analyses

Statistical analyses were done using a computer programme (Minitab for Windows). Three-way Analysis of Variance (ANOVA) were used to test the significance of main and interactive effects of the cations on ethanol production in synthetic molasses and synthetic malt wort. Similarly, two-way ANOVA were used to test the main and interactive effects of K and Ca under low, intermediate and high levels of Mg . The quadratic response surface function (Khuri and Cornell, 1987) was then used to predict

the cation combinations that maximise ethanol production in the two media types under investigation as follows:

• **Response surface function for molasses wort fermentations**

For each level of Mg^{2+} :

$$\text{Alcohol} = \beta_0 + \beta_1 K + \beta_2 Ca + \beta_{11} K^2 + \beta_{22} Ca^2 + \beta_{12} K*Ca$$

•**Response surface function for Malt wort fermentations**

For each level of Zn^{2+} :

$$\text{Alcohol} = \beta_0 + \beta_1 Mg + \beta_2 Ca + \beta_{11} Mg^2 + \beta_{22} Ca^2 + \beta_{12} Mg* Ca$$

Where { β } are unknown parameters estimated from the data.

Separate regression analyses under the three different levels of Mg were conducted and the equations estimated from the data were subjected to ANOVA in order to test the suitability of the models. Similar data anlyses were conducted to study the suitability of the model estimated for synthetic malt wort fermentations . Separate response surfaces for ethanol production under low, intermediate and high levels of magnesium in molsses wort and similar levels of zinc in malt wort were estimated and plotted in 3-dimensions using the above response functions (Figs.4.1- 4.6). Further, the data on cellular cation analyses were subjected to ANOVA in order to study the influence of external cations on the intracellular levels and interactions in synthetic molasses media.

4.3. Results and Discussion

Influence of extracellular cations on ethanol and biomass production in synthetic molasses and malt wort

Results of the fermentation studies carried out in synthetic molasses are shown in Table 4.3. Analysis of variance of the data (Table 4.4) showed a very highly significant ($p<0.01$) influence of all three cations on ethanol production. The influence of the above cations on biomass production (Table 4.5) indicates that the intermediate levels of these cations in fermentation media caused higher cell biomass production. Analysis of

variance (Table 4.6) of the biomass data showed that the major effects of Ca^{2+} and the interactive effects of Ca^{2+} and Mg^{2+} did not cause significant influences on biomass production. However, K^{+}, Mg^{2+} and their interactions showed very highly significant ($p<0.01$) influences on biomass production. Similar studies carried out in the synthetic malt wort have shown a positive influence of zinc and magnesium on fermentation (Table 4.7) and ANOVA results (Table 4.8) revealed that there are highly significant ($p<0.01$) main effects of Zn^{2+}, Mg^{2+} and Ca^{2+} on ethanol production.

Table 4.3 Alcohol production (%v/v) in synthetic molasses at different combinations of K^{+}, Mg^{2+} and Ca^{2+}

	High K^{+}			**Intermediate K^{+}**			**Low K^{+}**		
Mg^{2}	**Ca^{2+}**			**Ca^{2+}**			**Ca^{2+}**		
	High	**Int**	**Low**	**High**	**Int**	**Low**	**High**	**Int**	**Low**
High	5.92	5.91	6.98	7.04	7.09	7.54	7.49	7.56	6.61
Int	5.76	6.99	6.98	6.93	7.05	7.51	7.06	7.48	6.35
Low	5.50	7.01	6.71	6.76	7.05	7.21	7.21	6.73	5.94

Int = intermediate

Table 4.4 Analysis of variance of K^+, Mg^{2+} and Ca^{2+} effects on ethanol production

S.V.	DF.	S.S	M.S	F.	P
K	2	4 905	2.452	228.7	***
Ca	2	1.181	0.590	54.95	***
Mg	2	0.596	0.298	27.4	***
K*Ca	4	7.805	1.951	181.44	***
K*Mg	4	0.902	0.225	20.99	***
Ca*Mg	4	0.667	0.166	15.52	***
K*Ca*Mg	8	1.403	0.175	16.31	***
Error	27	0.290	0.010		
Total	53	17.753			

S.V.(source of variance), DF.(degrees of freedom).

M.S(means squares), S.S. (sums of squares), F. (variance ratio), P = probability, *** ($p<0.01$)).

Fermentation studies were conducted to study the major and interactive effects of K^+, Mg^{2+}, and Ca^{2+} on ethanol and biomass production. Treatments were allocated according to a 3^3 factorial design.

Table 4.5 Yeast biomass production (mg/l) at different combinations of cations K^+, Mg^{2+} and Ca^{2+}

	High K^+			Intermediate K^+			Low K^+		
Mg^{2+}	Ca^{2+}			Ca^{2+}			Ca^{2+}		
	High	Int	Low	High	Int	Low	High	Int	Low
High	24.5	26.5	31.3	36.5	36.0	34.5	37.0	39.5	31.5
Int	31.5	28.5	23.5	25.0	28.0	36.0	41.5	31.0	26.5
Low	25.0	27.5	28.0	26.0	28.5	28.0	32.0	27.5	25.5

Int = Intermediate

Table 4.6 Analysis of variance of K^{2+}, Mg2+and Ca^{2+}effects on yeast biomass production

Source	DF	SS	MS	F	P
K	2	246.68	120.91	20.41	0.00***
Ca	2	17.74	10.34	1.74	0.19
Mg	2	267.86	129.43	21.85	0.00***
K*Ca	4	268.72	62.66	10.58	0.00***
K*Mg	4	121.49	30.89	5.22	0.00***
Ca*Mg	4	47.04	11.07	1.87	0.14
K*Ca*Mg	8	281.62	35.20	5.94	0.00***
Error	27	154.00	5.92		
Total	53	1405.17			

DF = degrees of freedom, SS = Sums of squares, MS = mean square, F = F-distribution, P = probability, NS = not significant, * = p <0.10, * * = P.0.05.

Fermentation experiments were conducted to study the major and interactive effects of K^+, Mg^{2+} and Ca^{2+} on ethanol and biomass production. Treatments were allocated according to 3^3 factorial design.

Table 4.7 Alcohol production at different combinations of cations Zn^{2+}, Mg^{2+} and Ca^{2+}

	High Zn^{2+}			Intermediate Zn^{2+}			Low Zn^{2+}		
Mg^{2+}	Ca^{2+}			Ca^{2+}			Ca^{2+}		
	High	**Int**	**Low**	**High**	**Int**	**Low**	**High**	**Int**	**Low**
High	3.31	3.33	2.93	3.74	2.87	2.69	2.78	2.61	2.65
Int	3.12	2.80	2.94	3.06	2.75	1.58	2.97	2.42	2.38
Low	3.04	2.66	2.40	2.87	2.58	2.43	2.48	2.31	2.23

Int = Intermediate

Table 4 .8 Interaction between Zn^{2+}, Mg^{2+} and Ca^{2+} in synthetic malt wort

Analysis of variance for alcohol

Source	DF	SS	MS	F
Zn	2	1.60010	0.80005	65.68 **
Mg	2	1.67321	0.83660	68.68 **
Ca	2	2.11976	1.05988	87.02 **
Zn * Mg	4	0.09345	0.02336	1.92 NS
Zn * Ca	4	0.24049	0.06012	4.94*
Mg * Ca	4	0.04878	0.01219	1.00 NS
Zn * Mg Ca	8	0.62303	0.07788	6.39 **
Error	27	0.32887	0.01218	
Total	53	6.72769		

** Significant at $p<0.01$ level, * Significant at $p<0.05$ level, NS = Not significant.

Cation interactions and ethanol production in synthetic molasses wort

The ANOVA showed that under low levels (16 ppm) of magnesium there is a significant ($p<0.01$) effect of K^+ on the fermentation process. Under low Mg^{2+} levels, the main effect of Ca^{2+} also showed a significant ($p<0.01$) effect on alcohol production(Table 4.9). It is known that yeasts exhibit a higher affinity for Mg^{2+} than Ca^{2+} during fermentation (Fuhrman and Rothstein, 1968) and Mg^{2+} uptake by yeast cells may lead to media becoming deficient for Mg^{2+}. Ca^{2+} may also antagonise Mg^{2+} transport systems and this could explain the highly significant inhibitory influence of Ca^{2+} when fermentation is conducted under low levels of magnesium. The results of the analysis of variance under intermediate levels (70ppm) of Mg^{2+} (Table 4.10) show a significant ($p<0.01$) influence of K^+ on ethanol production by yeast. When there was a higher level (270ppm) Mg^{2+} in the medium the main effect of Ca^{2+} did not significantly influence ethanol production (Table 4.11). Wolniewicz *et al* (1988) showed that by altering the Mg^{2+}:Ca^{2+} ratio in favour of magnesium, alcohol production by yeast increased in molasses media, results recently verified by Walker *et al.* (1996). The analysis of the data of synthetic molasses wort fermentations using the 3-way ANOVA showed significant ($p<0.01$) two factor (K^+*Mg^{2+}), (K^+*Ca^{2+}), (Mg^{2+}*Ca^{2+}) and three factor (K^+*Mg^{2+}*Ca^{2+}) interactions. In order to fully investigate these interactions separate 3D response surface graphs were estimated. The different nature of the interaction for the chosen levels of Mg^{2+} can be clearly seen (Figs 4.1, 4.2 and 4.3). The response surfaces (Figs 4.1, 4.2, 4.3) show that ethanol production increases at intermediate levels of Mg^{2+} with increasing levels of K^+ and Ca^{2+} up to a certain level and then declines. Similarly, when high levels (270ppm) of magnesium were incorporated into the fermentation media, there were significant ($p<0.01$) effects of potassium. The non-significant main effect of Ca^{2+} when there was a high level of Mg^{2+} in the fermentation medium again suggests a higher affinity of yeast for Mg^{2+} over Ca^{2+}. This finding supports the views of Wolniewicz (1988) , Saltukoglu and Slaughter (1983) and Walker *et al* (1994).

Table 4.9 Two-way ANOVA, for the influence of K^+ and Ca^{2+} on ethanol production at low (16ppm) level of Mg^{2+}

S.V	DF.	S.S	M.S	F	P
K.	2	1.1063	0.5531	376.52	***
Ca	2	0.6127	0.3063	208.54	***
K*Ca	4	3.8035	0.9508	647.22	***
Error	9	0.0132	0.0014		
Total	17	5.5358			

Table 4.10 Two-way ANOVA, for the influence of K^+ and Ca^{2+} on ethanol production at intermediate (70ppm) level of Mg^{2+}

S.V	DF.	S.S	M.S	F.	P
K	2	1.0756	0.5378	62.98	***
Ca	2	1.0622	0.5311	62.20	***
K*Ca	4	2.6283	0.6570	76.95	***
Error	9	0.0768	0.6570		
Total	17	4.8430			

Table 4.11 Two-way ANOVA, for the influence of K^+ and Ca^{2+} on ethanol production at high (270ppm) level of Mg^{2+}

S.V	DF.	SS.	MS.	F.	P
K.	2	3.6265	1.8132	81.48	***
Ca	2	0.1744	0.0872	3.92	N.S
K*Ca	4	2.7767	0.6940	31.19	***
Error	9	0.2003	0.0222		
Total	17	6.7780			

N.S. (not significant).

Cation interactions and ethanol production in synthetic malt wort

The results of a 3-way ANOVA (Table 4.8) showed that there are significant major effects of the cations Zn^{2+} , Mg^{2+} and Ca^{2+} during malt wort fermentation. Although in molasses magnesium was considered as the most influential cation, in malt wort zinc was taken as the major cation of interest and further statistical analysis of data carried out under the influence of low (0.11ppm), intermediate (0.26 ppm) and high (0.48 ppm) levels of zinc. According to the results (Table 4.7), ethanol production increased with the increasing zinc concentration. Lomander (1965) has previously outlined the possible interaction between Zn^{2+} and Ca^{2+} during fermentation. However, the data analysis here did not show significant two factor interactions between Zn^{2+}*$Mg^{2+}$and Mg^{2+} * Ca^{2+}. This is clearly visible in the surface response curves (Figs. 4.4, 4.5 and 4.6). Compared with the interactive effects of zinc and manganese which share similar ligands N_2 and S , Mn^{2+} and Ca^{2+} share O_2 as a ligand. Therefore, Mn^{2+} may exert an indirect effect on the activity of Zn^{2+} and it could be visible as an interactive effect of $Zn^{2+}$and Ca^{2+}. Similarly, three-factor interactions (Zn^{2+}*Mg^{2+}*Ca^{2+}) in malt wort fermentations may be an indirect effect of the competition for a common ligand (O_2) with Mn^{2+} which influences the activity of Zn^{2+}. Studies conducted by Densky *et al* (1966) in brewing wort using ale yeast have shown a stimulatory effect of Zn^{2+}at a level of 0.1 to 1ppm. Further studies have shown that the speed of fermentation increases and fermentation times reduced by approximately 15-20% following zinc supplementations. Desmaretz (1993) showed that increased Zn^{2+} levels (0.45 ppm) promoted fermentation. Zinc plays a major role in fermentative metabolism since it is essential for the activity of zinc metalloenzymes like alcohol dehydrogenase, aldehyde dehydrogenase, cysteine desulphydrase etc. Zinc may also activate riboflavin synthesis, and increase protein content in fermenting yeast. It also has the capacity to stimulate the uptake of maltose and maltotriose into cells thus increasing the rate of fermentation.

Influence of extracellular cations on intracellular cation levels.

Cellular levels of the cations K^+, Mg^{2+}, Ca^{2+} were determined after digesting cells as described in the Methods section (2.2.5). The presence of high levels of intracellular K^+ ions shows the importance of this ion in the cell metabolism (Table 4.12). K^+ is essential for the uptake of $H_2PO_4^-$ for metabolism. Fermenting yeast cells uptake more K^+ than the respiring cells. According to the analytical results, celluar K^+ ions levels behaved independently irrespective of the K^+ levels in the media . Such behaviour was proven by the analysis of variance (Table 4.13) of the data which showed that the external K^+ had no significant influence on cellular K^+ levels. Further, these results indicate that yeast cells do not accumulate K^+ even though external medium is rich in K^+. However, according to the ANOVA, medium Mg^{2+} highly ($p<0.01$) influences internal K^+ levels. This could be due to the competition for transport sytems (Otero and Rayes, 1994) and binding sites and to extrusion of K^+ during the uptake of Mg^{2+}(Jones and Greenfield, 1984). These findings also support the idea of Jones and Greenfield (1984) who suggested a role of K^+ in regulating divalent cation transport in yeast by excreting $2K^+$ for each divalent cation taken up. Further, uptake of K^+ into cells is accompanied by extrusion of Na^+/K^+ ions and K^+ transport may be inhibited by the presence of other alkali metals. The results also indicate a significant ($p<0.10$) influence of Ca^{2+} on the cellular K^+ levels. Results also reveal interactive effects of Mg^{2+} and Ca^{2+} which show significant influence on cellular K^+ levels. Analytical results for cellular Mg^{2+} (Table 4.14) show that, unlike K^+, extracellular Mg^{2+} greatly influences the intracellular levels of Mg^{2+} demonstrating that yeast cells accumulate more Mg^{2+} when the growth medium is rich in this ion. This could be due to the importance of this ion for various cellular metabolic activities (Walker and Duffus, 1983; Lewis *et al*, 1978). Analysis of variance of the data (Table 4.15) shows very highly significant influences ($p<0.01$) of the other major cations (K^+, Ca^{2+}) on cellular Mg^{2+} levels. Intracellular Ca^{2+} levels are also influenced by external levels to some extent (Table 4.16). However, K^+ appeared not to significantly influence Ca^{2+} levels compared with Mg^{2+}.

The interactive effects of K^+ with Ca^{2+} , and Ca^{2+} with Mg^{2+} showed significant ($p<0.05$ and $p<0.10$ respectively) influences on celluar Ca^{2+} levels (Table 4.17).

Table 4.12 Intracellular potassium(fg/cell) at different levels of extracellular K^+, Mg^{2+} and Ca^{2+}

	High K^+			Intermediate K^+			Low K^+		
Mg^{2+}	Ca^{2+}			Ca^{2+}			Ca^{2+}		
	High	Int	Low	High	Int	Low	High	Int	Low
High	253.0	291.1	241.5	163.0	235.1	252.1	189.7	242.2	212.7
Int	296.2	291.1	310.5	248.8	262.2	395.5	258.7	292.1	272.7
Low	207.8	226.5	163.5	208.3	200.0	152.4	98.1	286.5	142.9

Table 4.14 Intracellular magnesium (fg/cell) at different levels of extracellular cations K^+, Mg^{2+} and Ca^{2+}

	High K^+			Intermediate K^+			Low K^+		
Mg	Ca^{2+}			Ca^{2+}			Ca^{2+}		
	High	Int	Low	High	Int	Low	High	Int	Low
High	674.5	636.1	496.1	529.9	553.6	421.2	582.2	463.0	506.8
Int	331.1	200.2	127.1	106.7	123.5	359.8	283.8	93.9	107.5
Low	315.5	176.8	221.9	151.9	124.9	191.6	163.7	181.4	47.0

Table 4.16 Intracellular calcium (fg/cell) at different levels of extrcellular cations K^+, Mg^{2+} and Ca^{2+}

	High K^+			Intermediate K^+			Low K^+		
Mg	Ca^{2+}			Ca^{2+}			Ca^{2+}		
	High	Int	Low	High	Int	Low	High	Int	Low
High	437.9	307.6	160.2	341.2	280.6	275.2	275.1	275.0	227.6
Int	500.3	434.0	163.7	438.3	327.6	206.3	330.8	386.7	253.9
Low	513.0	255.9	160.4	512.6	205.1	232.3	440.0	319.1	235.4

Table 4.13 Analysis of variance for intracellular K^+ at different levels of extracellular K^+, Mg^{2+} and Ca^{2+} at the end of fermentation of synthetic molasses

Source	DF	SS	MS	F	P
K	2	12561	6280	1.78	0.188 NS
Ca	2	20709	10354	2.94	0.070*
Mg	2	85880	42940	12.17	0.000***
K*Ca	4	21504	5376	1.52	0.233 NS
K*Mg	4	3520	880	0.25	0.907 NS
Ca*Mg	4	32739	8185	2.32	0.083 *
K*Ca*Mg	8	25280	3160	0.90	0.534 NS
Error	27	95247	3528		
Total	53	297439			

DF = degrees of freedom, SS = Sums of squares, MS = mean square, F = F-distribution, P = probability, NS = not significant, * = $p<0.10$, * * = P.0.05.

Table 4.15 Analysis of variance for intracellular Mg^{2+} at different levels of extracellular K^{+}, Mg^{2+} and Ca^{2+} at the end of fermentation of synthetic molasses

Source	DF	SS	MS	F	P
K	2	73837	36919	28.12	0.00***
Ca	2	51661	25831	19.67	0.00***
Mg	2	1546388	773192	588.87	0.00***
K*Ca	4	93856	23464	17.87	0.00***
K*Mg	4	12439	3110	2.37	0.07 NS
Ca*Mg	4	30954	7738	5.83	0.00***
K*Ca*Mg	8	108661	13583	10.34	0.00***
Error	27	35451	1313		
Total	53	1953243			

DF = degrees of freedom, SS = Sums of squares, MS = mean square, F = F-distribution, P = probability, NS = not significant, * = p<0.10, * * = P.0.05.

Table 4.17 Analysis of variance for intracellular Ca^{2+} at different levels of extracellular K^+, Mg^{2+} and Ca^{2+} at the end of fermentation of synthetic molasses

Source	DF	SS	MS	F	P
K	2	7892	3946	0.73	0.49 NS
Ca	2	452387	226194	41.91	0.00***
Mg	2	43273	21637	4.01	0.03**
K*Ca	4	79920	19980	3.70	0.01**
K*Mg	4	9560	2390	0.44	0.77 NS
Ca*Mg	4	67925	16981	3.15	0.03*
K*Ca*Mg	8	16513	2064	0.38	0.92 NS
Error	27	145733	5398		
Total	53	823202			

DF = degrees of freedom, SS = Sums of squres, MS = mean squre, F = F-distribution, P = probability, NS = not significant, * = p <0.10, * * = P.0.05.

Predicting fermentation performance

The significant 3-way interactions between Mg^{2+}, K^+, and Ca^{2+} in synthetic molasses wort imply that combinations of K^+ and Ca^{2+} for maximising alcohol production depend on the level of Mg^{2+}. Hence we need to attempt to establish optimal combinations of K^+ and Ca^{2+} for low, medium and high levels of Mg^{2+} seperately. Similarly, in synthetic malt wort, the production of ethanol critically depends on the level of Zn^{2+}. For each experiment we established the maximum point on the quadratic response surface (see below) for the medium and high levels of Mg^{2+} (in molasses) and Zn^{2+} (in malt wort). Several workers have tried different methods to optimise nutrient levels in growth and fermentation media. These include elemental mass balances and programmed search techniques (Egli and Fiechter, 1980; Prokop *et al,* 1976). However,

in none of these studies are there clear indications that the statistical significance of the interactive effects of the main cations were considered and no attempt was made to predict the potential ethanol production from media with known cation concentrations.

The quadratic response model showed a high coefficient of determination (r^2 = 83.5, 83.3 and 86.7) under, high intermediate and low levels of Mg^{2+}, respectively, and the overall regression model was statistically significant (each $p<0.01$, Tables 4.18 - 4.20) . Simple calculus showed that respective combinations of : $K^+/Mg^{2+}/Ca^{2+}$ at 2154/270/1118, 5102/70/509 (ppm) may maximise ethanol production. Experimental ethanol yields of 7.88 , 7.08 (% v/v), were close to the predicted values of 7.75 , 7.49 (%v/v), respectively (Table 4.21) at these cation combinations (Figs.4.1, 4.2 and 4.3): each were within their appropriate 95% prediction interval. Following the same approach, test fermentations were conducted in authentic molasses wort (21° Brix) with known concentrations of $K^+/Mg^{2+}/Ca^{2+}$. The predicted and actual ethanol levels are shown in Table 4.22 . It is clear that actual values are within the 95% prediction interval for Sri Lankan, and Javan cane and Danish beet molasses but were too low for Pakistani, Brazilian and Mexican and South African cane molasses. This could be due to the very high levels of K^+ and Ca^{2+} found in those latter media.

Table 4.18 Analysis of Variance for the regression equation under low (16ppm) levels of Mg^{2+}

Alcohol = $1.66 + 3.10K + 2.68Ca - 0.491K^2 - 0.375Ca^2 - 0.621K*Ca$

Source of variance	DF	SS	MS	F
Regression	5	4.80053	0.96011	15.67**
Error	12	0.73536	0.06128	
Total	17	5.53589		

Since the regression is significant ($p<0.01$) it concluded that the model is a suitable for predictions.

Table 4.19 Analysis of Variance for the regression equation under intermediate (70ppm) levels of Mg^{2+}

Alcohol = 5.06 + 3.399K + 6.18Ca - 1.85K^2 - 5.81Ca^2 - 3.82K*Ca

Source of variance	DF	SS	MS	F
Regression	5	4.03649	0.80730	12.01**
Error	12	0.80660	0.06722	
Total	17	4.84309		

Analysis shows the regression is significant ($p<0.01$) so that the model is suitable for the prediction of cation combinations.

Table 4.20 Analysis of Variance for the regression equation under high (270ppm) levels of Mg^{2+}

Alcohol = 5.45 + 4.40 K + 2.34 Ca - 2.41 K^2 + 1.22 Ca^2 - 3.78 K*Ca

Source of variance	DF	SS	MS	F
Regression	5	5.6571	1.1314	12.11**
Error	12	1.1209	0.0934	
Total	17	6.7780		

The regression is significant, ($p<0.01$) so that the model is suitable for the predictions

Table 4.21 Predicted cations combinations and ethanol yields in synthetic molasses

Cation combination K/ Mg/ Ca (ppm)	Predicted yield (%v/v)	Actual yield (%v/v)	Predictive interval 95%
2154/270/1118	7.75	7.88	6.90 - 8.61
5102/70/50	7.49	7.08	6.85 - 8.12
7231/14/1006	5.63	5.50	4.95 - 6.75

Table 4. 22 Fermentation of Authentic molasses (21°Brix)

Molasses	Cations(ppm) K/Ca/Mg	Predicted EtOH(%v/v)	Actual EtOH(%v/v)	Prediction interval(95%)
Sri-Lankan	2000/400/160	7.15	6.62	6.55 - 7.74
Brazilian	1100/600/210	7.13	6.45	6.55 - 7.77
Javan	1500/200/50	6.23	6.44	5.63 - 6.84
Danish(Beet)	2200/230/23	6.52	6.83	5.95 - 7.10
Mexican	1600/600/60	6.88	6.20	6.28 - 7.48
Pakistani	2500/600/160	7.47	4.61	6.88 - 8.07
S. African	3500/600/200	7.60	6.33	7.02 - 8.18

For the malt wort experiments the quadratic response surface models showed a high coefficient of determination (r^2 = 86.3, 87.8 and 81.9, Tables 4.23 - 4.25)) under high, intermediate and low levels of Zn^{2+}, respectively. Further, the overall regression model was statistically significant, (each $p<0.01$). Simple calculus showed that respective combinations of : $Zn^{2+}/Mg^{2+}/Ca^{2+}$ at 0.1/100/350, 0.2/300/300 and 0.4/350/105ppm predict ethanol production levels of 3.3, 4.69 and 3.49 respectively (Table 4.26). Experimental ethanol yields of 3.43 , 3.34 and 3.32(% v/v), were close to the predicted values of 3.3 ,4.69 and 3.49 (%v/v), respectively, at these cation combinations (Figs. 4.4, 4.5 and 4.6). Following the model, test fermentations were conducted in two types of authentic malt wort with known concentrations of $Zn^{2+}/Mg^{2+}/Ca^{2+}$. Results (Table 4.27) show that the actual values were always higher than those predicted. This could be due to extraneous factors (eg. chelators, growth factors) present in authentic wort which may influence yeast metabolism.

According to results of experimental fermentations and consequent data analysis, it is possible to come to the following conclusions regarding cationic interactions in molasses and malt wort fermentations. Firstly, in molasses fermentations there are significant main effects of K^+, Mg^{2+} and Ca^{2+} on ethanol production. Significant two-factor (K^+*Mg^{2+}, K^+*Ca^{2+} and $Mg^{2+}*Ca^{2+}$) and three-factor ($K^+*Mg^{2+}*Ca^{2+}$) interactions on fermentation at each level of Mg^{2+} studied have been revealed. The authentic media studies proved the potential use of response surface curves to predict ethanol production for a given combination of cations. Secondly, malt wort fermentation

studies revealed that Zn^{2+}, Ca^{2+} and Mg^{2+} have significant influences on fermentation whilst Zn^{2+} and Ca^{2+} exert a significant interaction during fermentation for each level of Zn^{2+} studied. Although the three-factor interaction, (Zn^{2+}*Mg^{2+}*Ca^{2+}) was significant, the estimated response surface predictions did not match with the actual values for the authentic malt wort fermentations.. This could be due to non-significant interactions between Mg^{2+}*Zn^{2+} and Ca^{2+}*Mg^{2+}. This matter should be further studied before reaching any conclusions regarding the applicability of statistical predictive modelling in malt fermentations.

Table 4.23 Analysis of Variance for the regression equation under high (0.4ppm) levels of Zn^{2+}

Alcohol = 2.30 + 0.00454 Mg-ppm + 0.00779 Ca-ppm - 0.000005 Mg-ppmsq - 0.000023 Ca-ppmsq-0.000009 Mg.Ca. R.sq = 86.3%

Source	DF	SS	MS	F
Regression	5	1.25925	0.25185	15.11**
Error	12	0.20001	0.01667	
Total	17	1.45925		

The regression is significant ($p<0.01$) so that the model is suitable for the predictions

Table 4.24 Analysis of Variance for the regression equation under intermediate (0.2ppm) levels of Zn^{2+}

Alcohol = 2.44-0.00056 Mg-ppm + 0.00439 Ca-ppm +0.0000008 Mg-ppmsq-0.000014 Ca-ppmsq +0.000019 Mg.Ca. R-sq = 87.8%

Source	DF	SS	MS	F
Regression	5	2.39793	0.74959	17.19**
Error	12	0.33475	0.02790	
Total	17	2.73268		

The regression is significant, ($p<0.01$) so that the model is suitable for the predictions

Table 4.25 Analysis of Variance for the regression equation underlow (0.1ppm) levels of Zn^{2+}

Alcohol = 2.11 + 0.00480 Mg-ppm + 0.00087 Ca-ppm - 0.000012 Mg-ppm-sq + 0.000005 Ca-ppm-sq - 0.000003 Mg.Ca. R-Sq = 81.9%

Source	DF	SS	MS	F
Regression	5	0.76599	0.15321	10.84**
Error	12	0.16966	0.01414	
Total	17	0.93566		

The regression is significant, ($p<0.01$) so that the model is suitable for the predictions

Table 4.26 Predicted cation combinations and ethanol yields in synthetic malt wort

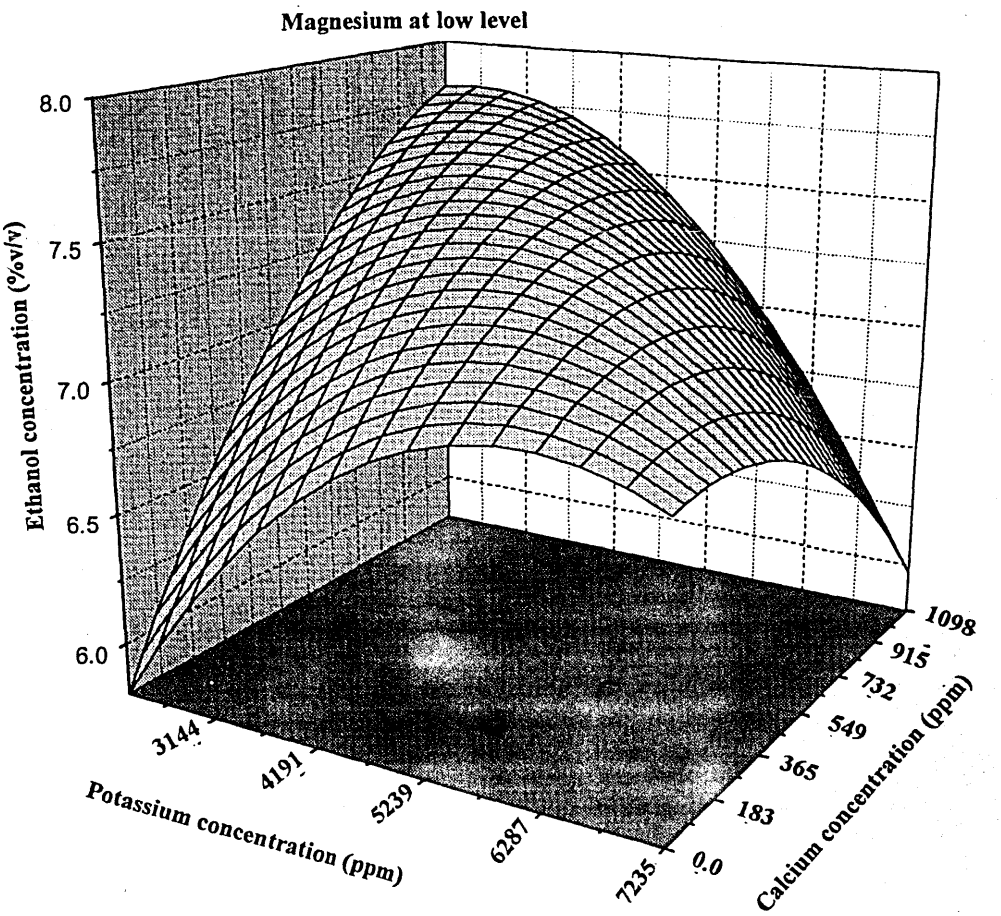
Cation combination Zn/ Mg/ Ca (ppm)	Predicted yield (%v/v)	Actual yield (%v/v)	Predictive interval 95%
0.1/100/350	3.33	3.43	1.93 - 4.74
0.2/300/300	4.69	3.34	3.20 - 6.19
0.4/350/105	3.49	3.32	2.49 - 4.5

Table 4. 27 Fermentation of authentic malt wort (OG 1040)

Malt wort	Cations(ppm) Zn/Ca/Mg	Predicted EtOH(%v/v)	Actual EtOH(%v/v)	Prediction interval(95%)
Lab. extract	0.18/55/84	2.74	4.23	2.37 - 3.11
Lab.ext.(alt)	0.34/55/134	3.05	4.39	2.68 - 3.43
Brewery	0.24/60/105	2.92	4.59	2.54 - 3.30
Brewery (alt)	0.44/60/155	3.05	4.98	2.66 - 3.44

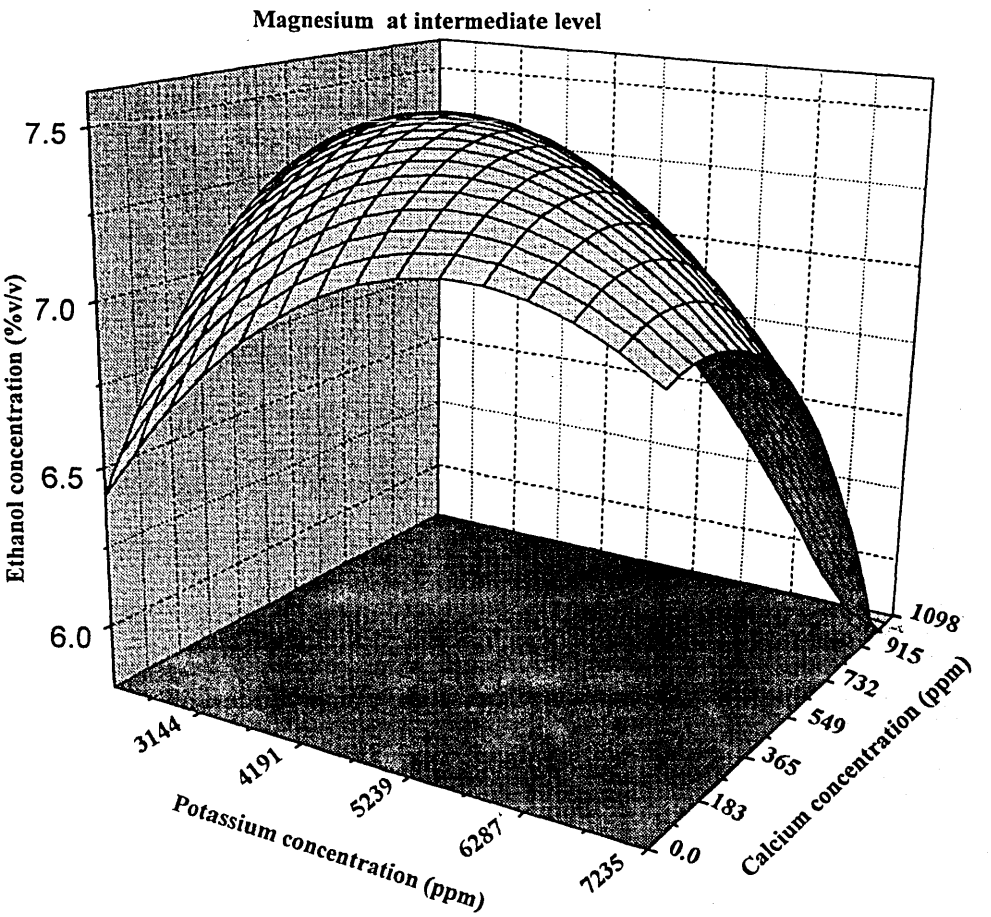
alt (altered)

Fig. 4.1 Response surface plot for the fitted value levels of ethanol concentration (%v/v) at various levels of K^+ and Ca^{2+} at a low level of Mg^{2+} in synthetic molasses.



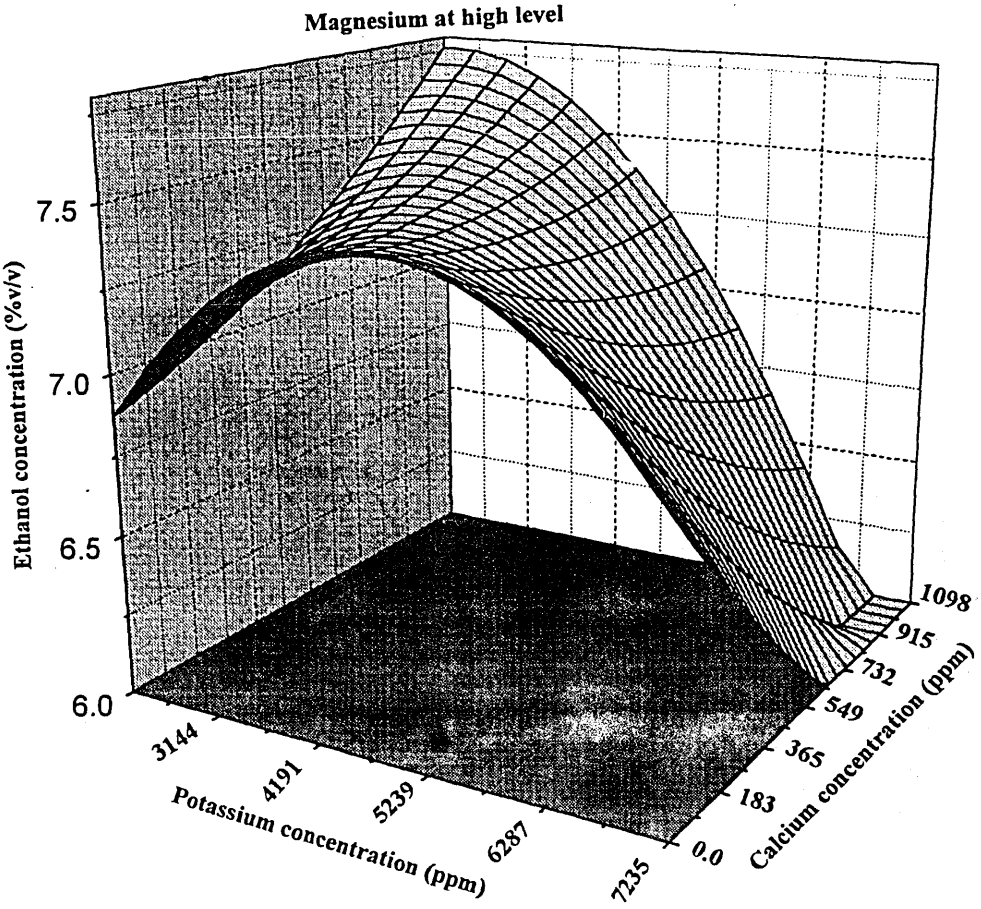
At low K^+ values increasing Ca^{2+} leads to an increase in ethanol. At intermediate K^+ values increasing Ca^{2+} from low to intermediate values increases ethanol; further addition of Ca^{2+} leads to a reduction in ethanol. At higher K^+ values increasing Ca^{2+} from low to intermediate values has only a small effect on ethanol levels; further increasing Ca^{2+} levels reduces ethanol concentration.

Fig. 4.2 Quadratic response surface for the fitted value of ethanol concentration (%v/v) at varying levels of K^+ and Ca^{2+} at an intermediate level of Mg^{2+} in synthetic molasses.



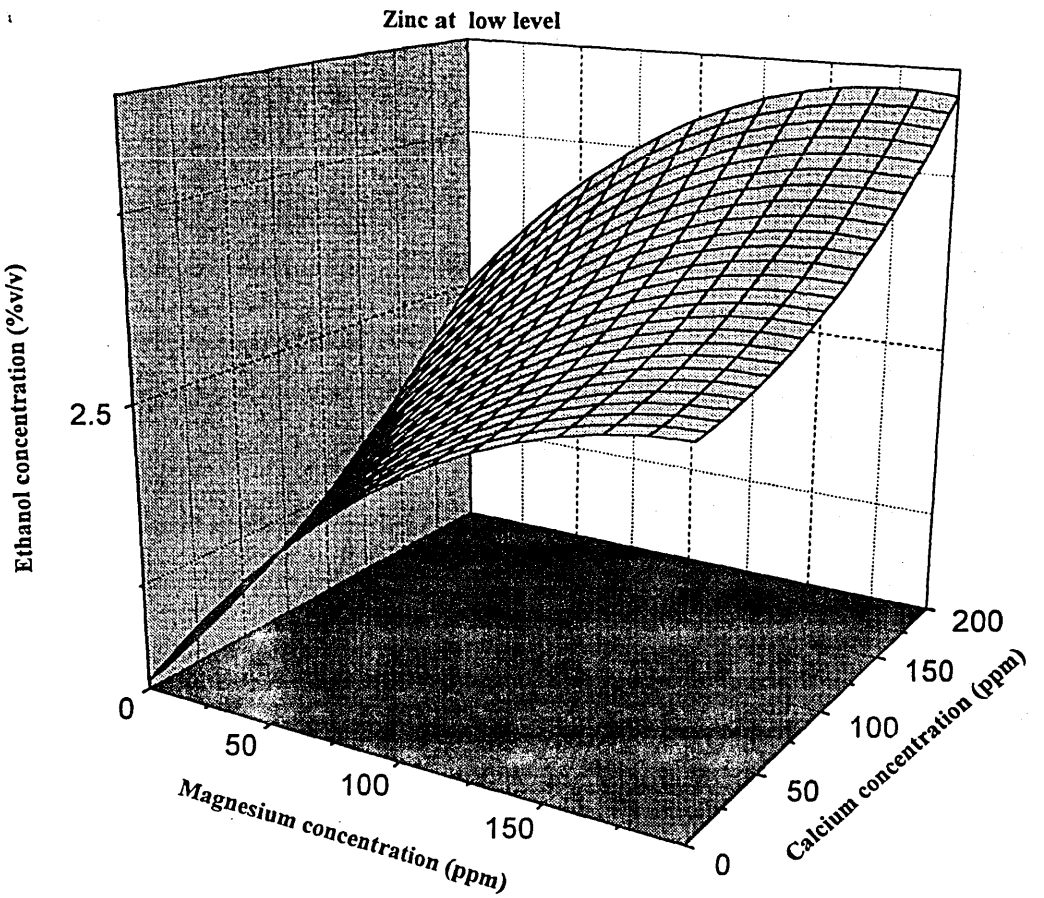
For all levels of K^+ increasing Ca^{2+} from very low levels increased ethanol concentration. However, further increasing Ca^{2+} leads to a reduction in ethanol concentration. The response surface has a maximum value of 7.49 %v/v when K^+ = 5702 and Ca^{2+} = 509. Experiments at this level had a mean ethanol concentration within the 95% confidence interval for this combination.

Fig. 4.3 Quadratic response surface for the fitted value of ethanol concentration (%v/v) at varying levels of K^+ and Ca^{2+} at a high level of Mg^{2+} in synthetic molasses.



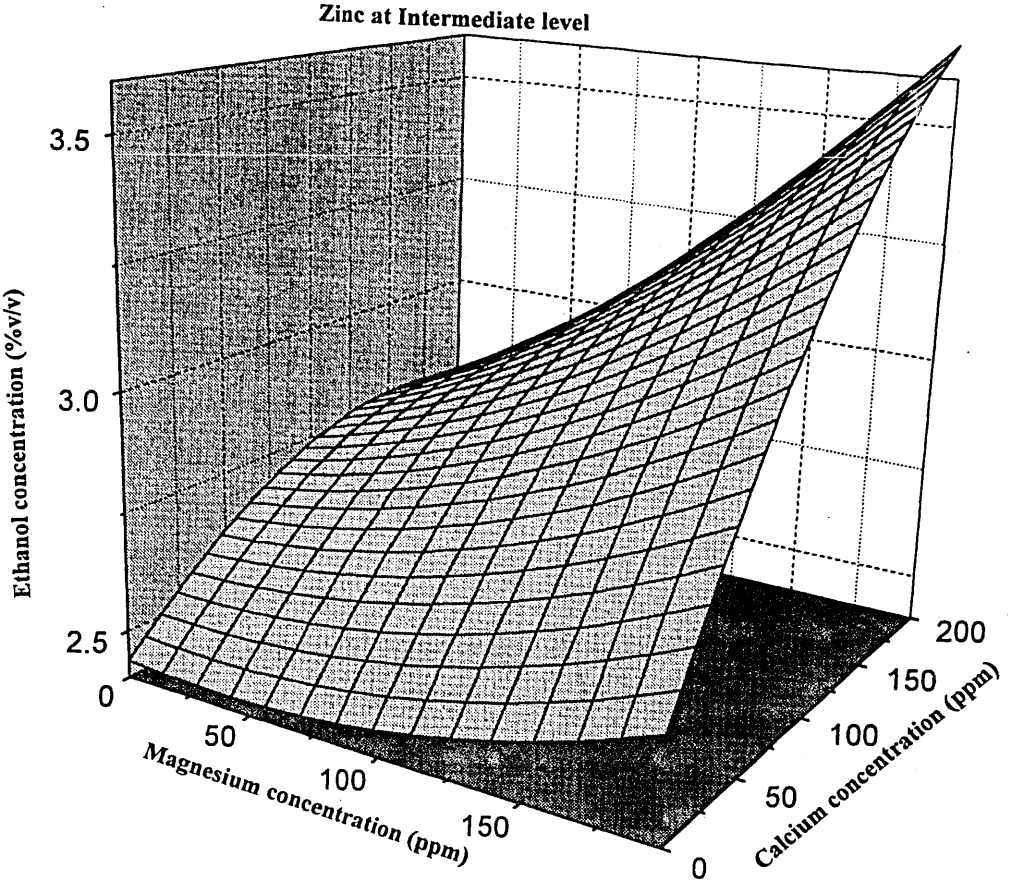
At low levels of K^+ and Ca^{2+} there is a sharp increase in ethanol production. Although further increases of K^+ show a stimulatory effect, Ca^{2+} exerts a negative influence on ethanol production. The response surface has a maximum value of 7.75(%v/v) when K^+ = 2154 ppm and Ca^{2+} = 1118 ppm . Experiments at this level had a mean ethanol concentration within the 95% confidence interval for this combination.

Fig. 4.4 Quadratic response surface for the fitted value of thanol concentration (%v/v) at variyng levels of Mg^{2+} and Ca^{2+} at a low level of Zn^{2+} in synthetic malt wort.



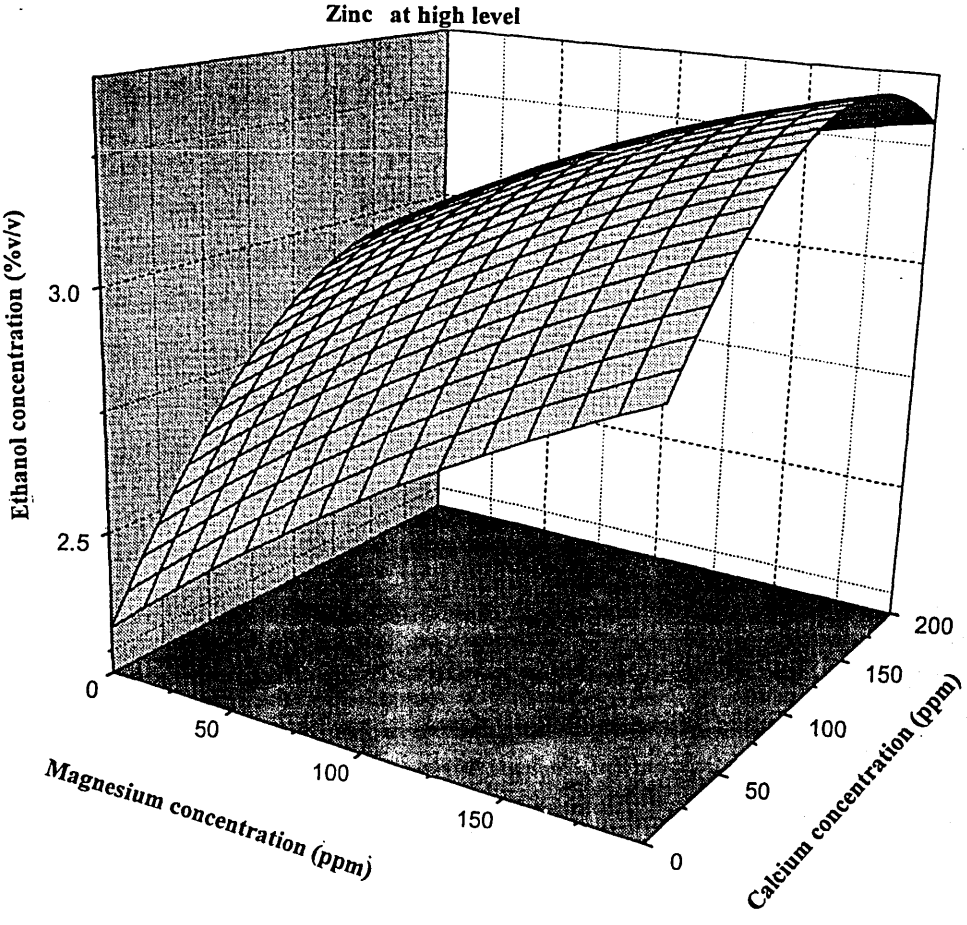
At low levels of Mg^{2+} and Ca^{2+} ethanol production is low. With the increasing levels of Mg^{2+} and Ca^{2+} ethancl level increases. The effect of Ca^{2+} seems to be higher at lower levels of Mg^{2+} in the medium. The response surface has a maximum value of 3.34 (%v/v) when Mg^{2+} = 100 ppm and Ca^{2+} = 350 ppm. Experiments at these levels had a mean ethanol concentration within the 95% confidence interval for this combination.

Fig. 4.5 Quadratic response surface for the fitted value of ethanol concentration (%v/v) at varying levels of Mg^{2+} and Ca^{2+} at an intermediate level of Zn^{2+} in synthetic malt wort.



According to the response curve at the low level of Mg^{2+} the ethanol production is low. However, with the increase of Mg^{2+} there is a slight increase in ethanol. Further, with the increase of Ca^{2+} concentration there is a higher increase of ethanol production. However, this increase changes with the changes of Mg^{2+} content in the medium. The response surface estimates a maximum of 4.6 (% v/v) for the combination Mg^{2+} = 300 and Ca^{2+} = 300ppm . The experimental value (3.34 %v/v) was within the 95% confidence interval for the combination.

Fig. 4. 6 Quadratic response surface for the fitted value of ethanol concentration (%v/v) at varying levels of Mg^{2+} and Ca^{2+} at a high level of Zn^{2+} in synthetic malt wort.



According to the response curve both increasing Mg^{2+} and Ca^{2+} levels increases ethanol production. However, when the Ca^{2+} level reached a certain level ethanol production declines even though the high level of Mg^{2+} shows further increase in ethanol production. The response surface has a maximum of 3.49 %v/v ethanol concentration for the combination Mg^{2+} = 350 and Ca^{2+} =105 ppm. An experimental value 3.32 %v/v was within the 95% confident interval.

Chapter 5. Metal Ions and Ethanol Tolerance in Yeast

5.1 Introduction

Ethanol is an end product of sugar fermentation by yeast . When it is accumulated in cells or in growth media , it acts as a chemical stress. Most studies carried out to date are based on the yeast species *S.cerevisiae* as it is widely used yeast for ethanol production. Several workers have defined ethanol tolerance of yeast in several ways and most of them are connected with the composition of fermentation media. Much of the interest on the physiological basis of ethanol tolerance in yeast has arisen from the work of Gray (1948) and Troyer (1953). Gray (1948) reported that ethanol tolerance was not limited to any one genus or species of yeast and that yeast cells were less tolerant to ethanol at high temperatures. He also found that ethanol tolerant yeast stored less lipid and carbohydrate materials than less tolerant yeast. Troyer (1955, cited by Stewart et al, 1988) confirmed Gray's findings; in addition he concluded that ethanol intolerance was not due to the fact that ethanol was a yeast metabolite, since methanol also inhibited sugar utilization in *S. cerevisiae.* The inhibitory effects of alcohols increases with the carbon number; lower, secondary alcohols are less inhibitory than their repective primary isomers. The work of Kalmokoff and Ingledew (1985, cited by Casey and Ingledew (1986) led to the conclusion that ethanol tolerance mainly depends on the composition of the medium than the genetics of the particular yeast strain. In their review on the ethanol tolerance of yeast Casey and Ingledew (1986) summarised that when fermentation media are rich in unsaturated fatty acids, sterols, proteins, amino acids, vitamins and metal ions yeast cells exhibit more tolerance to ethnol than media which is defficient in such nutrients. A number of inorganic ions act as macro and micro nutrients in yeast growth and fermentation. Inclusion of these ions in fermentation media therefore leads to improvements of fermentation performance in industrial situations. The role of these cations is in various capacities involving structural and enzymatic functions (Jones and Greenfield, 1984). Alteration of fatty acid compositon followed by

fluidization of membrane phospholipids due to ethanol accumulation is one of the main reasons for ethanol inhibition of growth and fermentation (Bevan *et al,* 1982). Because of this fundamental reason and other related alterations, yeast cell growth and growth kinetics, viability, morphogenesis, DNA synthesis (Berrah and Konetzka, 1962), membrane structure and function with mitochondrial mutations (Lester, 1965) and nutrient transport (Leao and Van Uden, 1984) are inhibited. All these impaired functions ultimately leads to the cesation of further ethanol production. However, several workers have shown that if fermentation media are rich in certain cations, those cations may bind to negative charges of phospholipids and mannan components and may act as shields to prevent ethanol toxicity. Further, the presence of cations like Ca^{2+} are known to induce stress proteins in the yeast cells to protect them from ethanol toxicity.

Supplementation of fermentation media with certain cations increases fermentation performance of yeast. For example, 0.5mM Mg^{2+} prolongs the decline of the exponential phase of yeast and adding Zn^{2+} increases fermentation progress with increased wort sugar uptake (Desmartez, 1993). Ethanol induced cation leakage reported in the ethanol producing bacterium *Zymomonas mobilis* is especially due to Mg^{2+} loss (Osman and Ingram, 1985). Addition of external Mg^{2+} minimises these leakage effects. Dasari *et al (*1990) have shown that Mg^{2+} could prevent ethanol toxicity whether from internal or external exposure. Deformation of mitochondria and low ethanol production in *S. pombe* could be overcome by Mg^{2+} -supplementation (Walker *et al,* 1982). Further, adding Mg^{2+} to molasses and malt wort has shown significant improvements in ethanol production by *S. cerevisiae* (see Chapter 3). Therefore, the following Chapter investigatated the protective effects of Mg^{2+} following addition of ethanol on yeast cell viability and ethanol production.

5.2. Experimental Approach

5.2.1. Effect of Mg^{2+} on the viability of yeast in the stationary growth phase

Yeast cultures

Saccharomyces cerevisiae distiller's yeast strain DCL'M' and *Kluyveromyces marxianus* were used for this study. Yeast cultures were grown on malt extact agar slants and stored at 4°C. Cultures were subcultured on agar slopes of the same medium and 24 hr actively growing cultures were used as intial cultures for inocula preparation.

Seed medium

Seed media (100ml each) were prepared in 500 ml deionised Erlenmeyer flasks, using modified Edinburgh Minimal Medium (EMM): glucose (50g), KCl (1g), $CaCl_2.6H_2O$(10mg), $MgSO_4.7H_2O$ (0.14g) together with micronutrients: H_3BO_3 (0.5mg), $MnSO_4.H_2O$ (0.4mg), $ZnSO_4.7H_2O$ (0.4mg), $FeCl_3.6H_2O$ (0.2mg), H_2MO_4. H_2O (0.16mg), KI (0.10mg), $CuSO_4.5H_2O$ (0.04mg) and citric acid (1.0mg), vitamins: inostol (10mg), nicotinic acid (10mg), calcium pantothenate (1mg), biotin (0.01mg), pyridoxine hydrochloride (0.04 mg), thiamine hyrochloride (0.04mg) and paraaminobenzoic acid (2mg) , ergosterol 2ml and ultrapure water (18MΩ) to 1000 ml. Steam sterilised (110°C) seed medium was inoculated with 24hr actively growing cultures and the flasks were incubated at 30°C in an orbital incubator at 150 r.p.m. until the cells reached stationary growth phase. Cells were then centrifuged, harvested, washed 4 times in ultrapure water and used for the inoculation of production media.

Production medium

Glucose (180g), $(NH_4)_2SO_4$ (5 g), $(NH_4)_2PO_4$ (1.42g), NaCl (0.5g), $MgSO_4.7H_2O$ (variable), $CaCl_2.6H_2O$ (10mg), KCl (1g), micronutrients: H_3BO_3 (0.5mg), $MnSO_4.H_2O$ (0.4mg), $ZnSO_4.7H_2O$ (0.4mg), $FeCl_3.6H_2O$ (0.2mg), H_2MO_4. H_2O (0.16mg), KI (0.10mg), $CuSO_4.5H_2O$ (0.04mg) and citric acid (1.0mg), vitamins: inostol (10mg), nicotinic acid (10mg), calcium pantothenate (1mg), biotin (0.01mg),

pyridoxine hydrochloride (0.04 mg), thiamine hyrochloride (0.04mg) and para-aminobenzoic acid (2mg), ergosterol and oleic acid mixture (2ml) and ultrapure water (18MΩ) to 1000 ml.

Experimental design and treatment combinations

The effect of five external ethanol levels (0, 5, 10, 15 and 17 %v/v) on *S. cerevisiae* cell viability in the presence of five Mg^{2+} levels (0, 20, 70, 270ppm) was studied. Treatments were allocated according to a randomised complete block design. The experiment was repeated using different ethanol levels (0, 4, 8, 12, 16 %v/v) and Mg^{2+} concentrations (0, 20, 70, 150ppm) Table 5.1 & 5.2.

Table 5.1 Treatment combinations used to study the influence of Mg^{2+} on yeast cell growth , viability and ethanol production.

Mg Levels (ppm)	**Ethanol concentration (%v/v)**				
	0	**5**	**10**	**15**	**17**
0	0/0	0/5	0/10	0/15	0/17
20	20/0	20/5	20/10	20/15	20/17
70	70/0	70/5	70/10	70/15	70/17
270	70/270	270/5	270/10	270/15	270/17

Table 5.2 Treatment combinations used to study the influence of Mg^{2+} on yeast cell growth and viability.

Mg Levels (ppm)	Ethanol concentration (%v/v)				
	0	**4**	**8**	**12**	**16**
0	0/0	0/4	0/8	0/12	0/16
20	20/0	20/4	20/8	20/12	20/16
70	70/0	70/4	70/8	70/12	70/16
150	150/0	150/4	150/8	150/12	150/16

Deionised, sterilized Erlenmeyer flasks (250ml) were used as batch fermenters with 100ml medium for the study. Initially variable ethanol concentrations were added into labeled flasks and followed by salt solutions, micronutrients, vitamins, ergosterol and varying concentrations of $MgSO_4.7H_2O$ solution to get 20, 70 and 150 and 270 ppm final magnesium ion concentrations. The flasks were then inoculated with a stationarry phase culture of *S. cerevisiae* harvested as above to get a final cell number of approximately 3×10^6 cells / ml. Upon inoculation, the flasks were incubated at 30°C in a rotary incubator 50 r.p.m.

Sampling

Samples were withdrawn from the fermenting broth at 0, 5, 18, 24, 48 and 72 hr intervals and total cell and viable cell numbers were counted using a haemocytometer; after staining the cells with methylene blue citrate (Figs 5.1 - 5.9). Ethanol production was also estimated using gas chromatography (Figs 5.13 - 5.16).

The experiment was repeated using the yeast *Kluyveromyces marxianus,* to evaluate the effects of ethanol levels (0, 4, 8, 12 and 16 %v/v) on cell viability in the presence of 0, 20, 70 and 150 ppm Mg^{2+} (Figs 5.10 - 5.12, 5.19, 5.20).

5.2.2. Effect of Mg^{2+}on the viability of yeast in exponential growth phase

Following the results of the previous experiment, it was decided to further investigate the effect of external ethanol on the viabilty of DCL'M' yeast cells which were in the exponential phase. For this study the seed culture was grown in the seed medium as previously and harvested after 8 hr prior to inoculation into experemental media and used for the experiment.

Production medium: This was similar to above experimental medium. However, the magnesium ion concentration and the ethanol concentration were varied. Initially, production medium was prepared as previously and 4 treatment combinations used in order to evaluate the effect of magnesium addition in the exponential phase of the growth. The treatment combinations were: Mg^{2+} (150ppm) and ethanol (10ml), Mg^{2+} (150ppm) and ultrapure water (10ml), Mg^{2+} (0) and ethanol (10ml), Mg^{2+} (0) and ultrapure water (10ml). After the initial preparation of the production media, above combinations of Mg^{2+}, ethanol and ultrapure water were added to deionised sterilized 250 ml Erlenmeyer flasks and production media were then incorporated to achieve the final volume of 100ml. A washed suspension of the seed inoculum which was in exponential phase was then inoculated to achieve a final cell density of 2×10^6 cells/ml.

Sampling

After inoculation viable cell numbers were taken using a Heamocytometer at 20 min intervals for 180 min. to completion. Results are summarised in Fig 5.18.

5.3. RESULTS AND DISCUSSION

Magnesium ions and ethanol stress in yeast

During the present study, it was decided to investigate the possible protective effects of Mg^{2+} against toxic effects of ethanol on the viability of *S.cerevisiae* and *K.marxianus.* According to the results of the control experiments conducted without added ethanol it is clear that yeast cells maintain higher levels of viability in the presence of increasing levels of Mg^{2+}. This finding is common for both *S.cerevisiae* and *K. marxianus.* Figs 5.1, 5.19, 5.20 and Table 5.3 show the influence of Mg^{2+} on cell viability of *S. cerevisiae* without added ethanol in the medium. All Mg^{2+} -supplemented cultures grew and multiplied to give very high cell numbers compared with Mg^{2+}-unsupplemented cells during the 72h experimental period. Initially, all levels of Mg^{2+} increased the viable cell number but at later stages of fermentation 20ppm Mg^{2+} appeared to be most effective. When the medium ethanol levels increased to 4 and 5%v/v yeasts further maintained their higher viabilities indicating the importance of Mg^{2+} in protecting cells from ethanol (Figs 5.2, 5.3, 5.18, 5.19 and Table 5.4). When yeast cells are under the influence of 4% ethanol, 150ppm Mg^{2+} and 70ppm Mg^{2+} showed protective effects in initial stages but 70 ppm Mg^{2+} showed protective effects in the later stages of fermentation. Yeast cells showed higher viabilties when the medium magnesium level was higher (270ppm) in the initial stages of fermentation in the presence of 5% external ethanol. Further increases of medium ethanol to 8-10%v/v showed a drastic reduction in viability in controls but Mg^{2+}-treated cells were capable of maintaining viability at a reasonable level (Figs 5.4, 5.5, 5.19, 5.20 and Table 5.5). In the presence of 8% ethanol, it was found that 150 and 70 ppm Mg^{2+} levels demonstrated the highest growth stimulatory effects. In the presence of 10% ethanol, 270 ppm Mg^{2+} showed stimulatory effects during the initial stages but 20 ppm was more effective latterly. Although a further increase of ethanol to 12-16%v/v resulted in complete loss of cell viability in Mg^{2+}-unsupplemeted media, Mg^{2+}-supplemented cells were able to maintain viability,

albeit at lower levels compared with previous ethanol levels (Figs 5.6, 5.7, 5.8 and Tables 5.6, 5.7) . The growth-inducing Mg^{2+} concentrations were 70ppm at the initial stage and 20ppm during the later stages. When cells were exposed to 15% ethanol, all Mg^{2+} levels apparently failed to maintain growth although some stimulatory effects were found with 20ppm Mg^{2+} present. Increasing ethanol concentrations to 17% (Fig 5.9) resulted in complete viability loss in all treatments. None of the Mg^{2+} levels could prevent cell death at this ethanol level.

Viability evaluations of *K. marxianus* in the above experiment revealed that, in similar fashion to *S.cerevisiae,* these cells also responded to the available Mg^{2+} by increasing viable cell number in ethanol-free treatments during the 72 h fermentation period. The stimulatory Mg^{2+} concentrations were 20 and 70ppm during the growth period (Fig 5.10 and Table 5.3). When medium ethanol level increased up to 4%, Mg^{2+}-unsupplemented cells showed a very high reduction in growth and viability whereas Mg^{2+} supplemented cells maintained a very high viabilty (Fig 5.11 and Table 5.4). Although *K. marxianus* is regarded as an ethanol intolerant yeast, this again demonstrates the protective effects of Mg^{2+} towards ethanol toxicity. When yeast cells are under the influence of 4%, ethanol 70 and 20ppm Mg^{2+} levels showed protective effects by increasing the viable cell number. However, further increases of ethanol up to 8% resulted in the complete loss of viabilty after 72 h, but cells were able to maintain their viability at lower levels up to 24 h in the Mg^{2+} supplemented treatments (Fig 5.12 and Table 5.5) . Further increases in ethanol concentration resulted in complete loss of viability even after 6h in the presence of Mg^{2+} but cells were able to maintain low viability after 1h following ethanol addition.

According to Stewart *et al* (1988), the possible target sites for ethanol in yeast cells are basically the yeast cell membranes which include: cell membrane, hydrophobic proteins of cell and mitochondrial membranes, nuclear membrane, vacuolar membrane,

endoplasmic reticulum, lysosomal membrane and hydrophilic proteins of the cytoplasm. According to the above results it is clear that the yeast cells exposed to ethanol in the presence of Mg^{2+} were capable of maintaining their growth and viability up to a certain level of ethanol compared with the controls. If the primary targets of ethanol are the yeast membranes, higher survival rates in Mg^{2+} -supplemented fermentations should be due to some form of protective effect of Mg^{2+} from ethanol toxicity or in other words, Mg^{2+} increases ethanol tolerance of yeast. The favourable response of *K.marxianus* to added Mg^{2+} is further evidence for the above view. Cell growth and viability measurements in the presence of ethanol are considered as a measure of ethanol tolerance (Stewart *et al*, 1987; Casey and Ingledew, 1986). This present findings support results of earlier workers on the importance of magnesium in cell growth and metabolism (Walker *et al,* 1990).

Table 5.3 Effect of Mg on cell viability of *S.cerevisiae* and *K. marxianus* grown in ethanol free media, after 48h

Magnesium concentration (ppm)	Viable cell Number (million/ml)	
	S.cerevisiae	*K.marxianus*
0	23.4	3.65
20	127	55.5
70	88.5	39.5
150	78.8	32.5

Table 5.4 Effect of Mg on cell viability of *S.cerevisiae* and *K. marxianus* grown in 4%v/v ethanol after 48h

Magnesium concentration (ppm)	Viable cell Number (million/ml)	
	S.cerevisiae	*K.marxianus*
0	21	12.8
20	56	62.2
70	131	47.8
150	169.2	54.6

Table 5.5 Effect of Mg on cell viability of *S.cerevisiae* and *K. marxianus* grown in 8%v/v ethanol after 48h

Magnesium concentration (ppm)	Viable cell Number (million/ml)	
	S.cerevisiae	*K.marxianus*
0	5	0
20	5.8	0
70	20	0
150	61.4	0

Table 5.6 Effect of Mg on cell viability of *S.cerevisiae* and *K. marxianus* grown in 12%v/v ethanol after 48h

Magnesium concentration (ppm)	Viable cell Number (million/ml)	
	S.cerevisiae	*K.marxianus*
0	3	0
20	6.6	0
70	6.4	0
150	4.2	0

Table 5.7 Effect of Mg on cell viability of *S.cerevisiae* and *K. marxianus* grown in 16%v/v ethanol after 48h

Magnesium concentration (ppm)	Viable cell Number (milllion/ml)	
	S.cerevisiae	*K.marxianus*
0	0.4	0
20	4.2	0
70	11.4	0
150	5.8	0

Fig 5.1

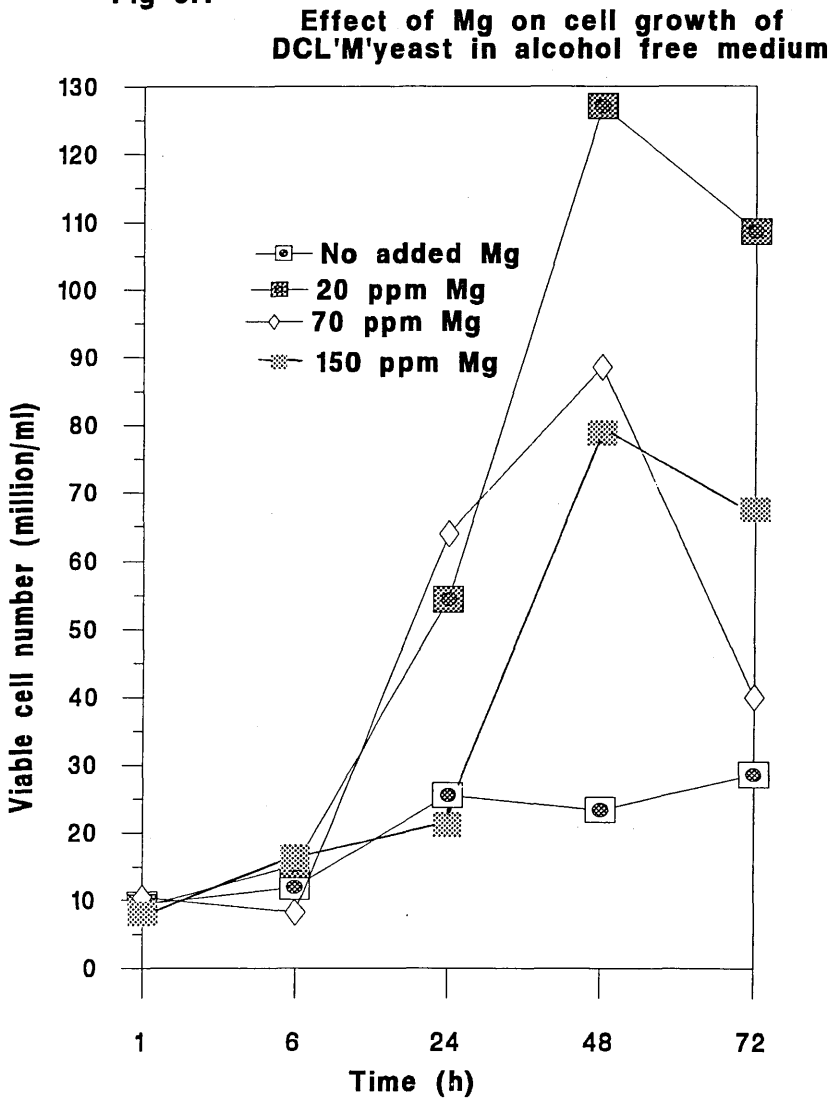

Effect of Mg on cell growth of
DCL'M'yeast in alcohol free medium
No added Mg
20 ppm Mg
70 ppm Mg
150 ppm Mg
Viable cell number (million/ml)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
1
6
24
48
72
Time (h)

Fig 5.2 Effect of Mg on cell growth of DCL'M' yeast in 4%v/v ethanol

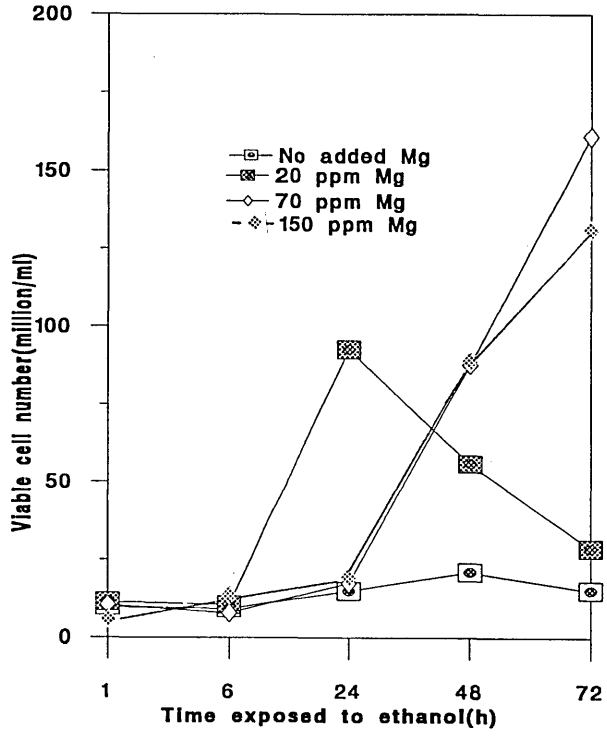


Fig 5.3 Effect of Mg on cell growth of DCL'M' yeast in 5%v/v ethanol medum

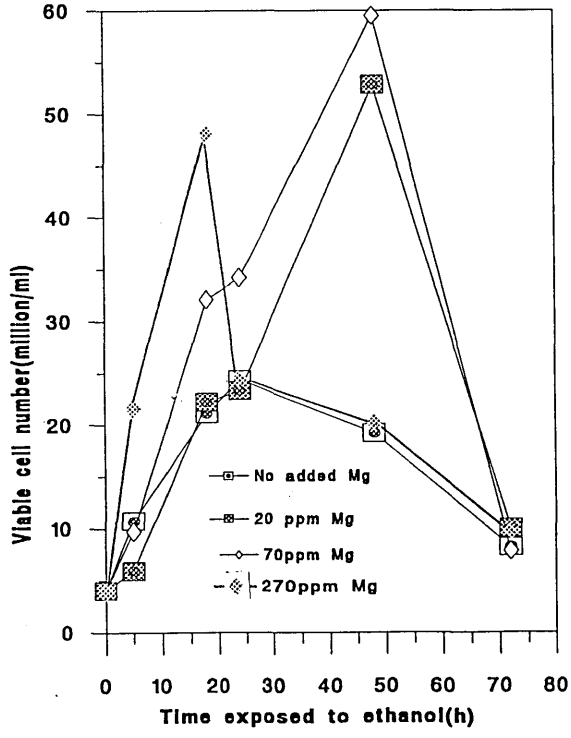


Fig 5.4 Effcet of Mg on cell growth of DCL'M' yeast in 8%v/v ethanol

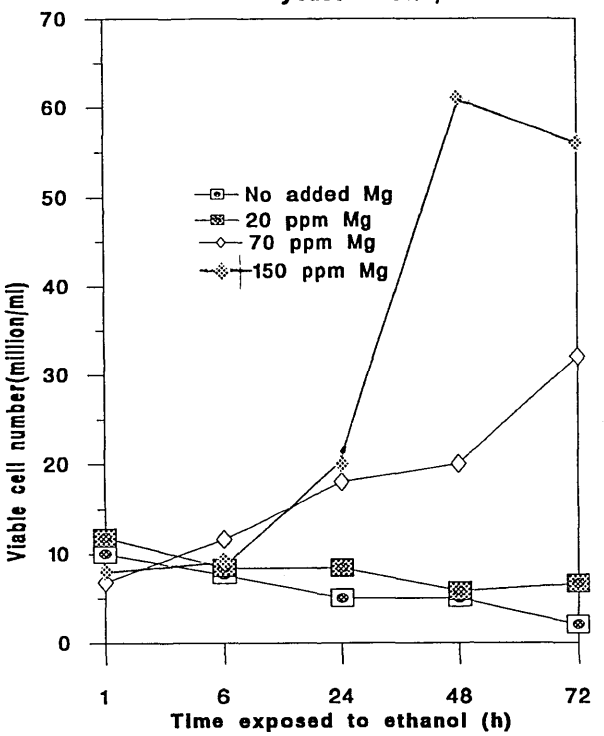


Fig 5.5 Effect of Mg on cell growth of DCL'M' yeast in 10%v/v ethanol

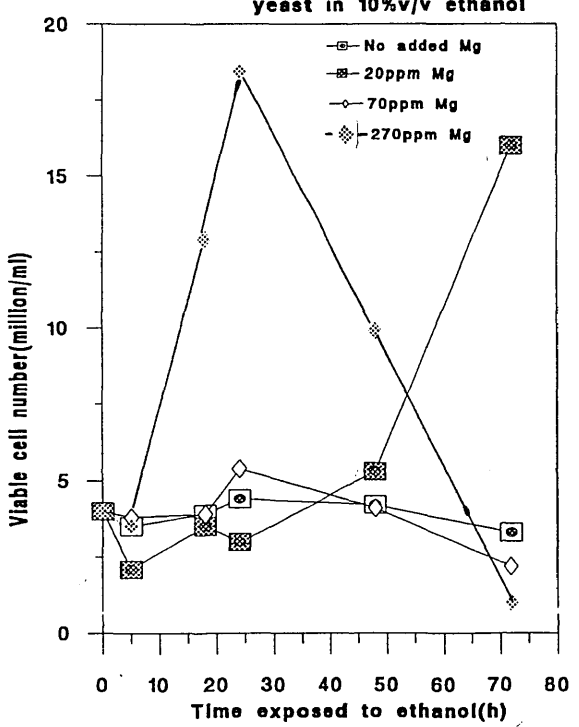


Fig 5.6 Effect of Mg on cell growth of DCL'M'yeast in 12%v/v ethanol

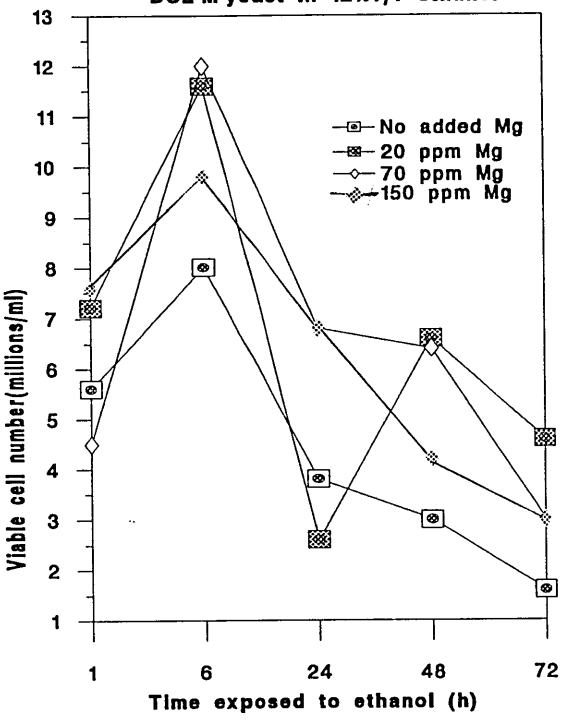


Fig 5.7 Effect of Mg on cell growth of DCL'M' yeast in 15%v/v Ethanol

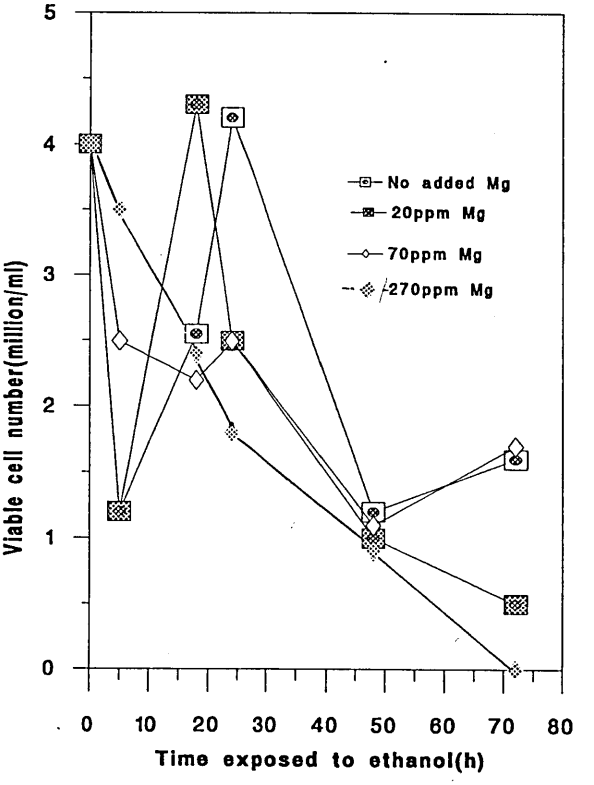


Fig 5.8 Effect of Mg on cell growth of DCL'M' yeast in 16%v/v ethanol

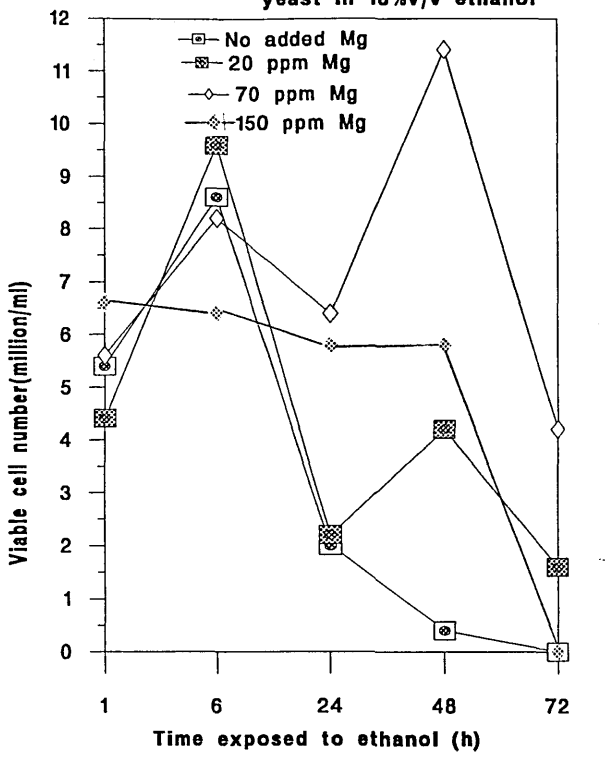


Fig 5.9 Effct of Mg on cell growth of DCL'M' yeast in 17%v/v ethanol medium

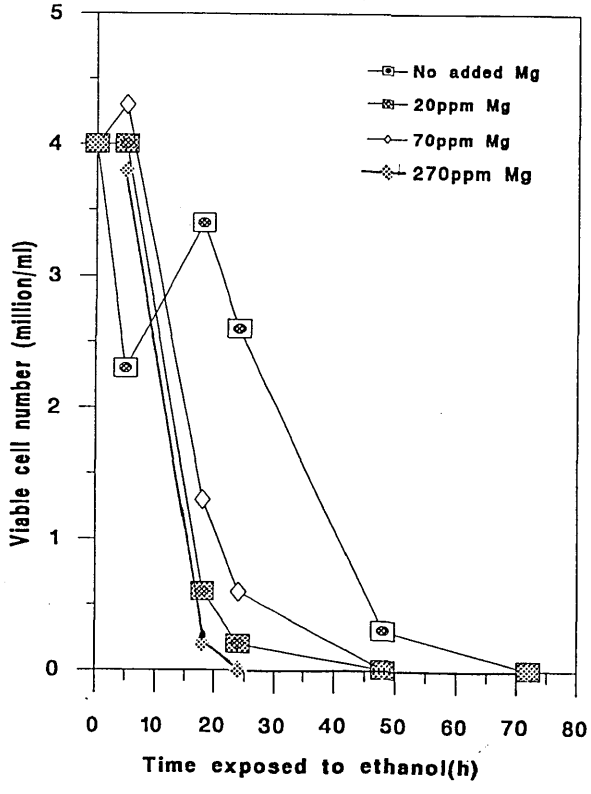


Fig 5.10

Effect of Mg on Growth of K.marxianus in ethanol free media.

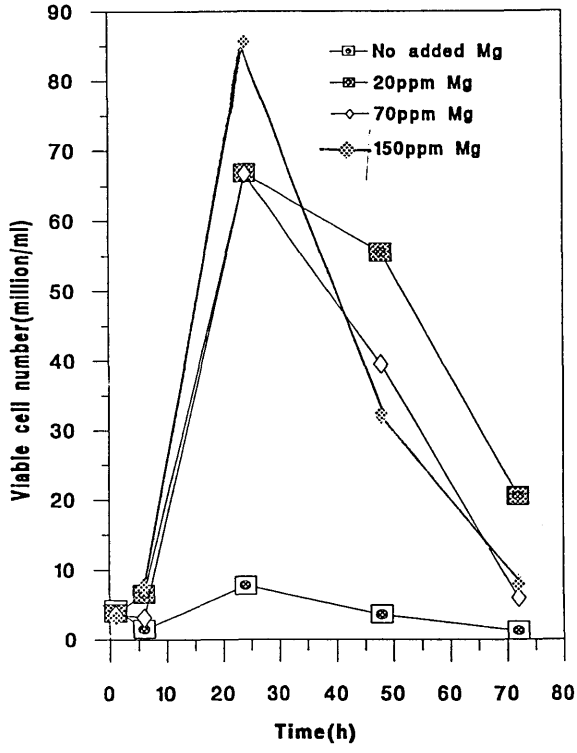


Fig 5.11

Effect of Mg on cell growth of K.marxianus in 4%v/v ethanol medium.

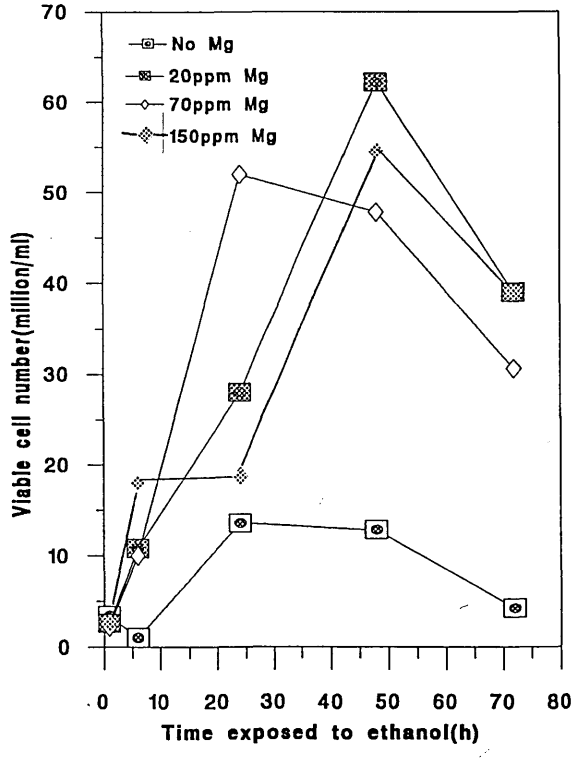
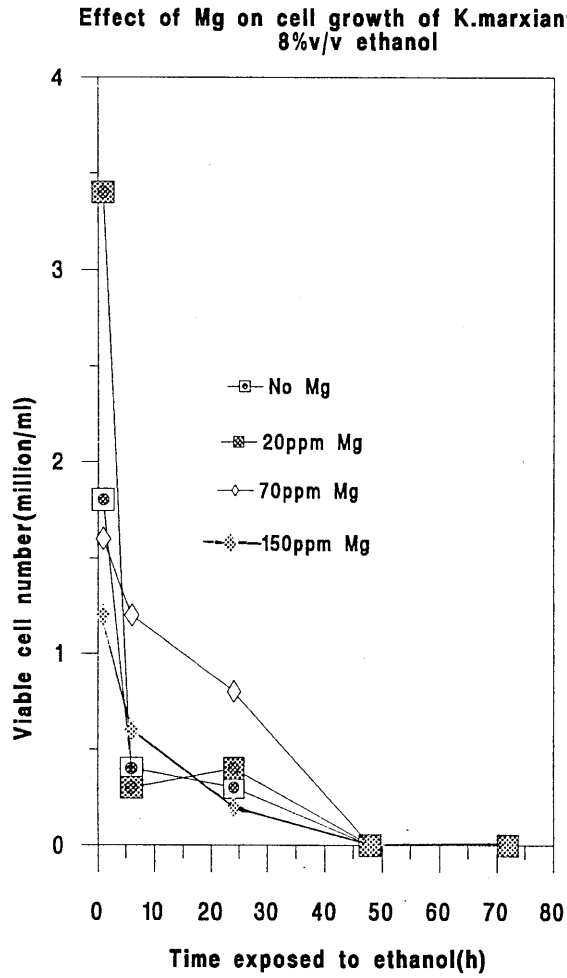


Fig 5.12


Effect of Mg on cell growth of K.marxianus in 8%v/v ethanol
Viable cell number(million/ml)
Time exposed to ethanol(h)
No Mg
20ppm Mg
70ppm Mg
150ppm Mg
0
1
2
3
4
0
10
20
30
40
50
60
70
80

Magnesium ions and fermentative activity in the presence of ethanol

Experimental findings on the influence of Mg^{2+} on ethanol-supplemented media are shown in Figs 5.13-5.17. The influence of Mg^{2+} on ethanol production in the absense of added ethanol is seen in Fig 5.13, which shows that with the increasing Mg^{2+} concentrations ethanol production increases during a 72 h fermentation period. When external ethanol is added into the fermentation medium, ethanol production differs between Mg^{2+}-supplemented and Mg^{2+}-unsupplemented cultures (Figs 5.14 - 5.16). Fig 5.17 shows the total ethanol in media under varing levels of ethanol after the fermentation period. When cells were deficient in Mg^{2+} , ethanol production was very low in ethanol-supplemented cultures. However, in the presence of Mg^{2+} cells produced an appreciable amount of extra ethanol and increases were related to the Mg^{2+} concentration. However, the increase was very low after the extracellular ethanol concentration exceeded 10%v/v, even in the presence of added Mg^{2+} in the medium.

Louereiro *et al* (1983, cited by Casey and Ingledew, 1986) showed that added ethanol is more toxic to yeast cells than produced ethanol since the latter is transported across the cell membrane. However, earlier workers showed that added ethanol was not as toxic as produced ethanol (Casey and Ingledew, 1986). This phenomenon depends on the substrate concentration According to Brown and Cook (1981) (cited by Casey and Ingledew, 1986), ethanol was found to have separate effects on yeast growth, fermentative activity, and cell viability. Cell viability was found to be extremely sensitive to ethanol inhibition with growth rate and especially fermentation rate being more resistant. Present experimental findings indicate protective effects of Mg^{2+} against chemical the stress of ethanol and support the findings of Dombek and Ingram (1986). According to the present results, *S.cerevisiae* cells were able to maintain high viability levels even in the presence of high concentrations of ethanol as long as cells were in contact with higher levels of Mg^{2+}. When the medium ethanol level increased from 0 - 17%, although there was a general trend of loss of viability in all treatments, there was

not a complete loss of viability when cells were treated with Mg^{2+}. Mg^{2+} un-treated cells lose viability with increasing ethanol levels through time. The loss of viability of yeast cells exposed to ethanol could be due to number of reasons, including: inhibition of glucose, maltose, ammonium, and amino acid uptake (Leao and Van Uden 1984) , enhanced passive influx of protons and decreased glucose-induced proton efflux , decreased intracellular pH, inhibition of plasma membrane ATPase activiity (Cartwright *et al*, 1986), increased membrane fluidity (Mishra, 1992), altered membrane fatty acid composition (Bevan *et al*, 1982), or leakage of amino acids, nucleosides, and nucleotides (Saguerio *et al*, 1988). As ethanol acts as an antimicrobial agent and a disinfectant, it directly influences the growth and survival of yeast cells when they are exposed to ethanol. However, the response varies with the yeast species and the culture conditions. For example, according to previous reports (eg. Mishra, 1992; and present studies) *S.cerevisiae* is more tolerant to ethanol than *K. marxianus*. Further, when media are rich in Mg^{2+} yeast cells demonstrate a higher tolerance to ethanol by maintaining their viability at an appreciable level and this is common for both *S.cervisiae* (known to be a yeast with high ethanol tolerance) and *K. marxianus* (an ethanol intolerant yeast). These findings support the views of Casey and Ingledew (1986) who reported media composition-dependent ethanol tolerance in yeast.

The reason for the capability of *S.cerevisiae* yeast cells to maintain viability even at very high levels of ethanol (16%v/v) could be due to the acummulation of the iso-enzyme cytrochrome P-450 which may also influence the oxidative removal of ethanol from the medium (Aoyama *et al,* 1984). The role of magnesium ions in the activity of cytochrome P-450 has yet to be studied. The data on the kinetics of cell multiplication in alcohol treated and untreated media shows that the yeast cells do not grow well without Mg^{2+} even in media which is rich in other nutrients. Walker and Duffus (1980) showed the absolute requrement of Mg^{2+} for cell division in the yeasts, *Schizosaccharomyces pombe* and *Kluyveromyces fragilis.* Gray (1941) was the first person to look at ethanol tolerance of yeast and his findings revealed that tolerance of yeast to ethanol is not

limited to one genus or species and that yeasts were less tolerant to ethanol at higher temperatures. According to Mishra (1992), several workers have reported the relationship between the kinetics of growth and the uptake of various nutrients in the presence of ethanol and Toda *et al* (1987 cited by Mishra, 1992) suggested that there is a critical concentration of ethanol above which yeast cells are unable to grow. This growth inhibitory concentration varies among yeast, ranging from 45g/L for *K.fragilis*, to 95g/L for *S.carlsbergensis*, to 125g/L for *S.cerevisiae*. According to the present results with added Mg^{2+} these limits have gone up to 63.2g/L for *K.marxianus* and 126.4g/L for *S.cerevisiae*. Since in this series of experiments Mg^{2+} was the only variable the results of the differences in the viabilities and ethanol production must be due to the sole influence of Mg^{2+}. Dasari *et al* (1989) found that Mg-supplemented yeast cells maintained higher viabilities in media with both endogenous and exogenous ethanol-supplemented media. As Stewart *et al* (1988) stated, membranes are the primary target of ethanol causing subsequent viability loss. According to Ingram and Buttke (1984), the inhibition of growth of *S.cerevisiae* by ethanol is caused at least in part by the interaction of ethanol with the plasma membrane. Alcohols also increase membrane permeability to ions and small metabolites in many types of cells and ethanol promotes the leakage of molecules from *S.sake* yeast (Ingram and Buttke, 1984). This could essentially lead to loss of viability of those cells. Increasing viability due to the increased availability of Mg^{2+} may therefore provide some form of cell membrane protection against ethanol-induced toxicity. Further, when ethanol was present in the medium, enzyme systems usually operate at below capacity rates. In *S.cerevisiae,* most of the ion transport is facilitated by three types of ATPases viz; plasma membrane, mitochondrial and vaculoar. Ethanol can inhibit the activty of these enzymes (Borst-Pauwels, 1981) which may lead to loss of viability and fermentative activity. However, these enzymes have an absolute requirement for Mg^{2+} and so beneficial and protective effects of this ion could be due to a direct stimulation of key enzyme activity.

Lipmann (1941) pointed out since about two high energy phosphate bonds are produced for every molecule of glucose fermented, the rate of dissipation of such bonds must balance the rate of their generation. Otherwise, the adenylic acid system may be saturated to such a degree that it can no longer serve as a phosphate acceptor in fermentative dephosphorylation. In general, syntheses and hydrolyses are the processes by which high energy bonds are depleted. Further, the experimental data indicated that in growing yeast cultures accumulation of high energy bonds is avoided through synthetic processes; the rate of glucose utilization is always higher during periods of active cell multiplication (Cartwright *et al,* 1987). The prevention of cell growth in accumulation of high energy phosphate bonds and resultant reduction of glucose utilization may appear as ethanol intolerance. When Mg^{2+} is present in the medium the accumulation of high energy bonds is essentially higher as it is involved in phosporylation processes. Increasing high energy reserves in cells increase the capacity of yeast cells to maintain their viability at higher levels under adverse environmental conditions like increased medium ethanol concentrations. Therefore, this evidence may provide an explanation for the protective effects of Mg^{2+} from ethanol toxicity.

The effect of adding Mg^{2+} and ethanol into yeast cells in the exponential phase is shown in Fig 5.18. According to the results yeast cells free of added ethanol continued growing exponentially without any disturbance upto 24 h This finding supports the findings of Dombek and Ingram (1986) who showed that Mg^{2+} supplementation prolonged the exponential phase of the growth of yeast. However, cells with 10% ethanol could not proceed into the exponential phase and ceased growth. This was recorded as a complete loss of viability after 24 h. The cells supplemented with 150ppm Mg^{2+} , however, maintained higher viabilites even after 32h, indicating the the long term protective effect of Mg^{2+} against ethanol toxicity.

Fig 5.13 Effect of Mg on Ethanol production in ethanol free media

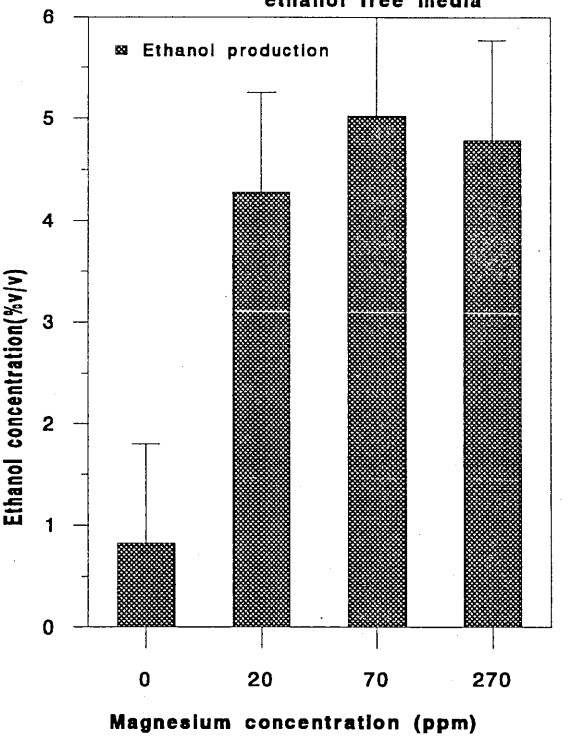


Fig 5.14 Effect of Mg on ethanol production in 5% Ehanol added media

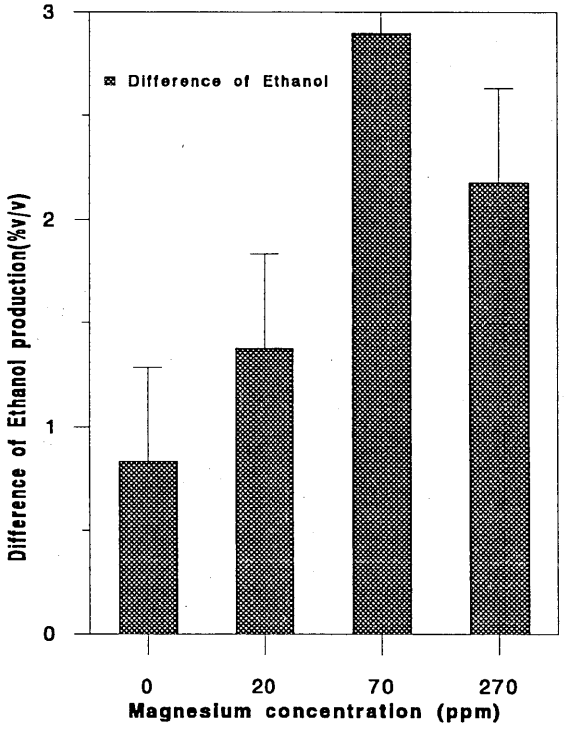


Fig 5.15 Effect of Mg on ethanol production in 10%v/v ethanol added medum.

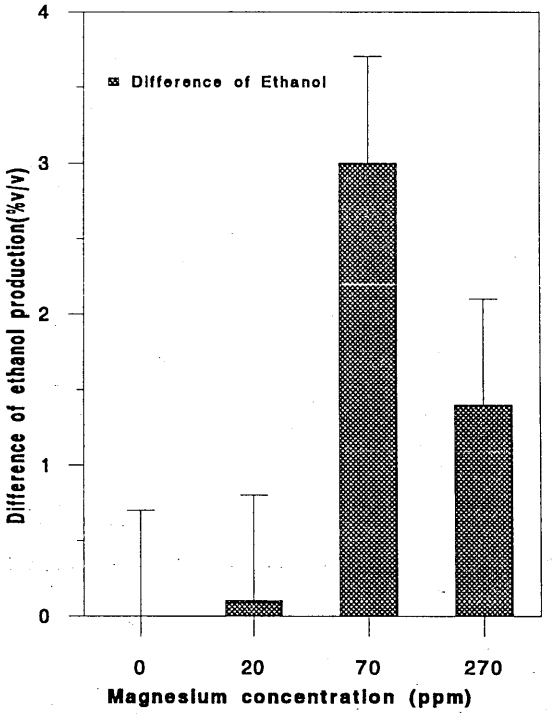


Fig 5.16 Effect of Mg on ethanol production In 15%v/v Ethanol added medium

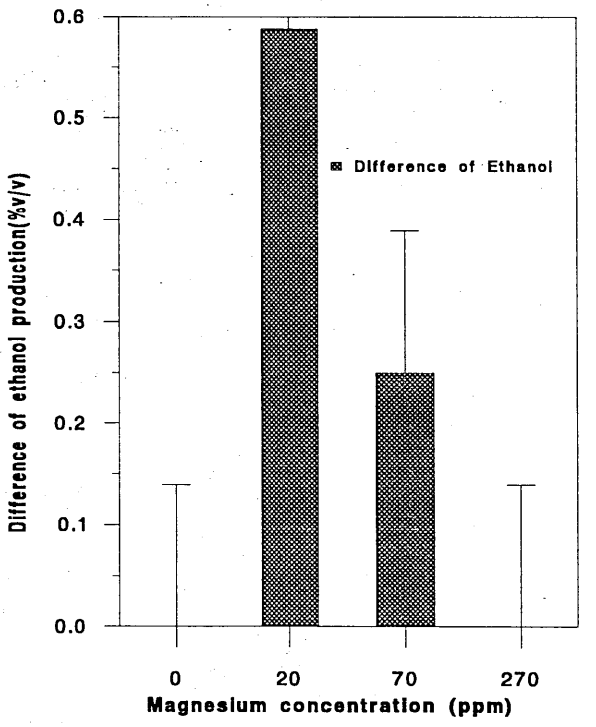


Fig 5.17 Ethanol production (%v/v) by DCL'M' yeast after 48h in Mg supplemented media

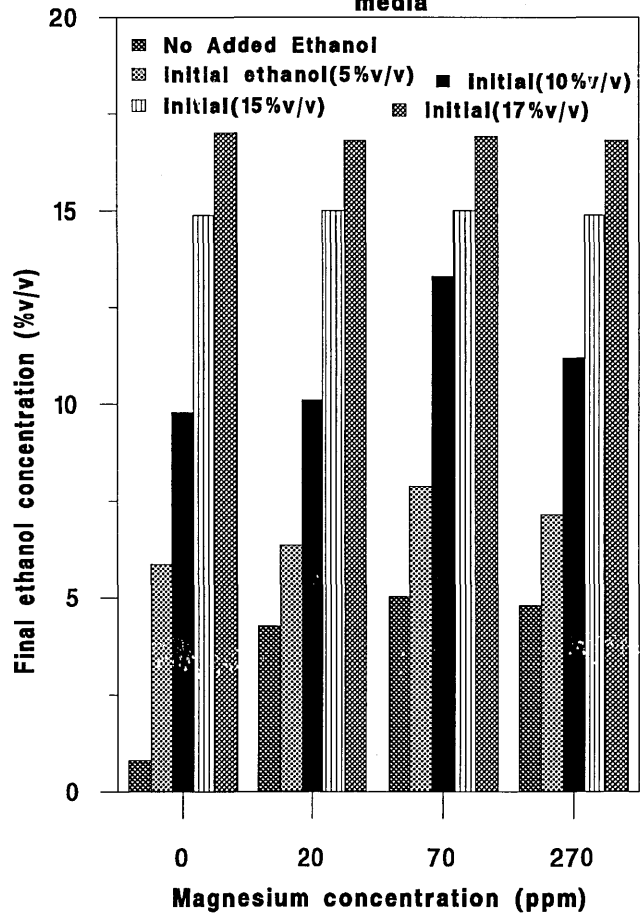


Fig 5.18 Effect of Mg and ethanol on growth of DCL'M' yeast in exponential growth phase

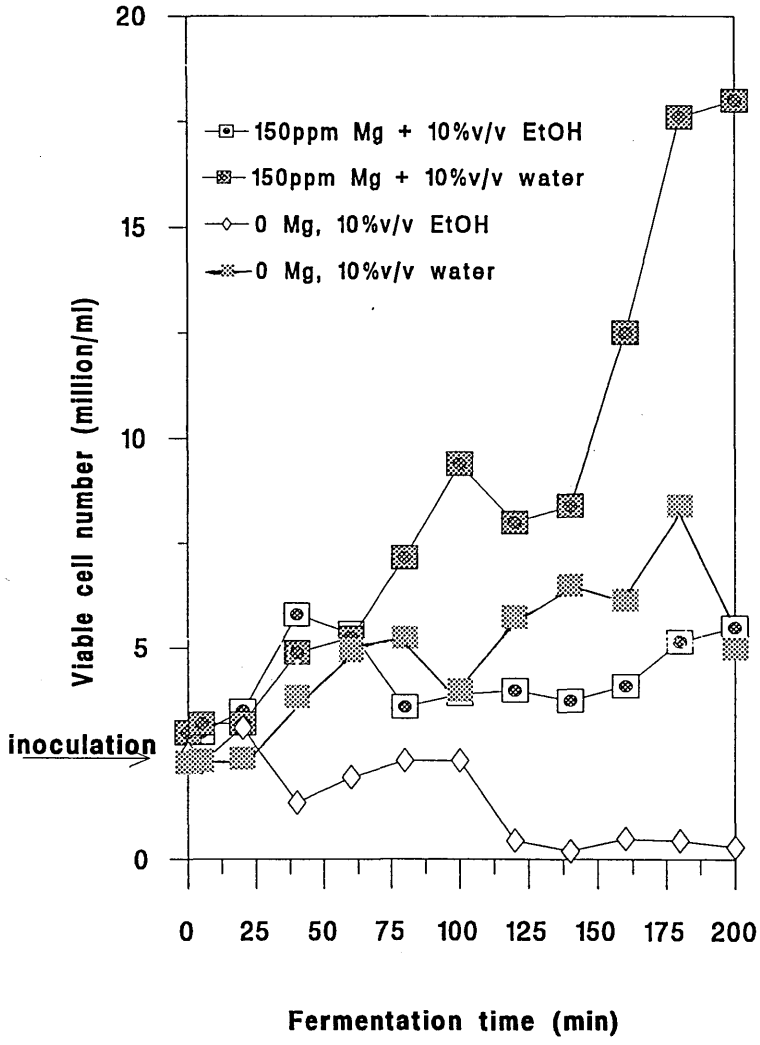


Fig 5.19 Long-term (24h) effect of Mg on cell growth of DCL'M' yeast in media containing varying levels of ethanol

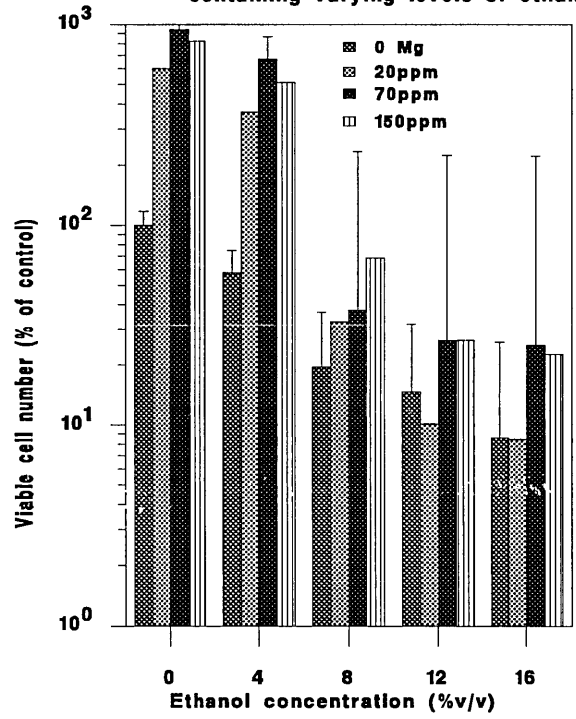
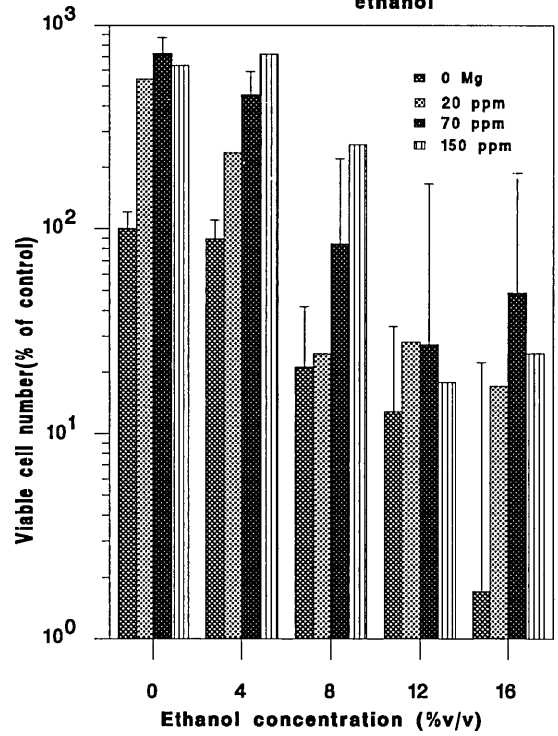


Fig 5.20 Long-term (48h) effect of Mg on cell growth of DCL'M' yeast in media containing varying levels of ethanol



Chapter 6: Concluding Discussion

Increasing energy prices have caused serious socio-economic problems throughout the world. Introduction of new energy producing technologies is therefore vital to overcome these problems. Use of biomass as an alternative source of energy to fossil oil is a partial but invaluable solution to this energy problem (Jackman, 1987). Readily utilised energy crop products or their by-products or less readily utilised forestry products and cellulosic wastes are certainly major candidates in this exercise. However, within a free market economy molasses, maize and cassava have been identified as the most economical basic raw materials. Fermentation production of ethanol from these sources has been practiced in many agricultural countries in the world and ethanol has become synonymous with renewable energy due to the economic possibilities of bioethanol as an octane-enhancer for petroleum fuels and as a replacement for these fuels (Casey and Ingledew, 1986). However, the conversion in the fermentation process and efficient energy consumption during distillation are vital for the economical viability of this industry. The nutritive qualities of the above raw materials directly influence the conversion efficiency of sugars to ethanol through fermentation as yeast requires a number of nutrients as cell constituents and co-factors of enzymes involved in their anabolic and catabolic metabolic process.

Results of experiments undertaken in this research show that media (molasses, malt wort and YEPD) are comprised of varing concentrations of several major cations: K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}. According to Otero and Reyes (1994), K^+, Mg^{2+} and Ca^{2+} are the most abundant cations in sugarcane molasses ranging from about 98% of the total metallic ions. Several other workers have supported this observations (Baker, 1979; MacWilliams 1968; Bovallius and Zacharias, 1971). K^+, Mg^{2+} and Zn^{2+} are recognized as major cations involved in yeast fermentation processes.

Synthetic media fermentation studies were carried out mimicking several levels (low, intermediate and high) of these cations in industrial media (molasses and malt wort). Results revealed that when the potassium concentrations increased the medium ethanol levels increased. However, this increase was limited up to 3500-5000ppm K^+ and further increases caused a decline in ethanol output. K^+ in fermentation has been shown by several workers to influence cation uptake, osmoregulation and the maintenance of cellular pH. Therefore, the experimental findings on the influence of K^+ could be due to one or several of the above. The influence of Ca^{2+} on alcohol production showed negative relationships with the increasing Ca^{2+} levels. This finding supports the views of Jones and Greenfield (1984) and the findings of Mairorella *et al* (1984) and Kyoko and Lewis (1971) who reported the inhibitory effects of Ca^{2+} on ATPase activity in yeast. Mg^{2+}, on the other hand, showed positive influences on ethanol production at all levels. This could be due to the essentiality of Mg^{2+} for yeast cell enzymatic regulation and macromolecular biosyntheses.

Variance analysis of the experimental results also revealed significant two (K^+*Mg^{2+}, K^+*Ca^{2+}, Mg^{2+}*Ca^{2+}) and three factor (K^+*Mg^{2+}*Ca^{2+}) interactions between these cations in terms of cell biomass production and ethanol production in synthetic molasses fermentations. These findings are supported by the experimental evidence of Nagamune and Inone (1981) who showed the existence of interactions between NH_4^+ and K^+ ions and Fe^{2+}, Zn^{2+} and Cu^{2+} during physiological activities of yeast in ethanol production in synthetic medium. The underlying cause for these interactions is basically the competition of cations for binding sites in the media, cell surfaces and the intracelluar milieu. Such competition dictates the bioavailability of these cations for metabolic processes (Hughes and Poole, 1981). As the interactions of these three cations is significant, changing concentrations of individual ions may affect the activity of other ions. Further to the above findings, it was also revealed that the main effect of Ca^{2+} was not significant on fermentation when a higher level of Mg^{2+} was present in the medium indicating that altering

the Mg^{2+}:Ca^{2+} ratio in favour of Mg^{2+} is one way of overcoming the problems caused by excess levels of Ca^{2+} in fermentation media. The beneficial effects of changing Mg^{2+}:Ca^{2+} ratios in fermentation media were shown previously by several workers (Saltukoglu and Slaughter, 1982, Walker *et al*, 1994; Wolneiewicz *et al*, 1988). When Mg^{2+} concentrations are higher in the medium the inhibitory concentrations of K^{+} and Ca^{2+} on ethanol production appear to be moved upward. Further, when medium Mg^{2+} was changed to intermediate and low levels these inhibitory effects of K^{+} and Ca^{2+} increase even at lower concentrations indicating their capability of blocking the availability of Mg^{2+} to yeast cells. These results confirm the possible interaction of K^{+} and Ca^{2+} with Mg^{2+} by competing for binding sites so that availability of Mg^{2+} for the fermentation process is curtailed leading to lower ethanol yields. In authentic molasses fermentations, therefore, it is possible to infer that these interactions may cause the unavailability of Mg^{2+} ions for yeast fermentations since molasses is rich in K^{+} and Ca^{2+} which definitely compete for binding sites and transport systems. The existence of a competition between Mg^{2+} and Ca^{2+}, Mg^{2+} and K^{+} have been shown by several workers (Saltukoglu and Slaughter, 1983; Wolniewicz, et al, 1988; Otero and Reyes, 1994). Variance analysis of synthetic malt wort experimental results revealed the existence of interactions between Zn^{2+}*Ca^{2+}, and three factor interactions between Ca^{2+}*Mg^{2+}*Zn^{2+}. However, it was not possible to confirm Ca^{2+}* Mg^{2+} or Mg^{2+}*Zn^{2+} interactions during synthetic malt wort fermentations as results of the variance analysis for these interactions were not statistically significant. Results therefore show that in malt wort existing Ca^{2+}:Mg^{2+} and Mg^{2+}:Zn^{2+} ratios were in favour of Mg^{2+} and neither Ca^{2+} nor Zn^{2+} could block the influence of Mg^{2+} on fermentation. The existence of higher levels of Mg^{2+} than Ca^{2+} and Zn^{2+} in authentic malt wort is known (MacWilliams, 1968; Jacobsen and Lie 1977). Further, the statistical significance of the interactions led to the estimation of quadratic response surface models for molasses fermentation, under low, intermediate and high levels of Mg^{2+} and malt wort fermentations, under similar levels of Zn^{2+}. Subsequent regression analysis showed models for both

synthetic molasses and malt wort were significant at all levels with higher coefficients of determinations r^2=83.3, 83.5, and 86.7, respectively above for synthetic molasses and r^2= 86.3, 87.8 and 81.9 for synthetic malt wort. Careful examination of the three dimensional graphics simulated from the estimates of the models made it possible to visualise the interactive behaviour of these cations during the fermentation process with respect to ethanol production. Thus, increasing K^+ under high levels of Mg^{2+} increases ethanol production upto a certain level whilst increasing Ca^{2+} showed deleterious effects on ethanol production in synthetic molasses. Similarly in malt wort, increasing Mg^{2+} under high levels of Zn^{2+} shows a positive influence on ethanol production whilst Ca^{2+} shows a negative effect. However, at the intermediate level of Zn^{2+} , Ca^{2+} acts synergistically to improve ethanol production at very low levels. Therefore, the influence of these cations varied with their availability in fermentation media. According to the model, predictive combination of $K^+/Mg^{2+}/Ca^{2+}$ at 2154/270/1118ppm appeared optimum for maximum ethanol production since predicted ethanol yields (7.75%v/v) matched actual yields (7.88%v/v) in the test fermentations for the synthetic molasses wort. The best-fit combinations of $Zn^{2+}/Mg^{2+}/Ca^{2+}$ for synthetic malt wort fermentation was 0.2/300/300ppm, and the actual ethanol yield (4.69%v/v) was within the confidence limits of the predicted yield (3.34%v/v). Predictions based on the compositions of authentic molasses were confirmed by fermentations and therefore it was possible to use the method for estimating ethanol yield from media with known levels of K^+, Mg^{2+} and Ca^{2+}. For malt wort fermentations, however, further studies are necessary before coming to any conclusions of the applicability of the method as predicted results did not match with the actual yields. Most industrial media including molasses and malt wort are composed of numerous organic constituents which could chelate or adsorb cations resulting in reduced availability of these essential cations for yeast to use in fermentation. Several workers have pointed out chelation, sequestration and adsorption of essential cations to various amino acids, proteins and polyphenolic compounds in molasses and malt wort (Jones and

Greenfield, 1984; Jacobsen and Lie, 1977). Therefore, it is possible to overcome this problem by relatively simple external supplementation of these cations in fermentation media.

Initial fermentation studies conducted in Mg^{2+}-supplemented South African molasses and YEPD broth using a wide range of yeast strains indicated the beneficial effect of Mg^{2+} irrespective of the yeast species or the strain used for fermentation. The yeast *Saccharomyces cerevisiae* distillers' (DCL'M') and wine strain (L2056) were the highest ethanol producers in those media. Favourable influences of external Mg^{2+} on fermentation by different yeast strains is a good example of the bio-unavailability of this essential cation in complex industrial and synthetic media. Variable ethanol yields between yeast species and strains could be due to differences in genetic characteristics of the yeasts.

Subsequent experiments conducted in Sri Lankan and South African molasses, YEPD broth and malt wort supplemented with a range of Mg^{2+} concentrations were carried out to evaluate several *Saccharomyces cerevisiae* industrial yeast strains (DCL'M', Saf- Levure and Red Star). Results again revealed the influence of strain variability on ethanol production. In Sri Lankan molasses yeast strains DCL'M' and Red Star produced highest ethanol levels (2.57, 2.33%v/v respectively, over the control) when molasses was supplemented with 100-150ppm of Mg^{2+}. Therefore, both yeasts are suitable for Sri Lankan molasses fermentation for industrial ethanol production. However, the suitability of the Red Star strain for potable alcohol production has to be further studied as it is a recommended baker's yeast. When South African molasses was supplemented with 150ppm Mg^{2+}, DCL'M' and Saf-Levure yeast produced the highest ethanol levels (1.21 and 1%v/v respectively over the control). However, the strain Red Star required a lower level (50ppm) of Mg^{2+} to achieve a yield of 2.14%v/v over the control. Mg^{2+}-supplementation at the rate of 100- 150ppm into YEPD broth resulted in enhanced ethanol

production at 1.21%v/v and 1%v/v over the controls by the strains DCL'M' and Red Star, respectively. However, the response of Saf-Levure to added Mg^{2+} was not remarkable. In malt wort DCL'M' was highly responsive to added Mg^{2+} (50-150ppm) by producing higher levels (over 1.5%v/v over controls) of ethanol. This yeast is commercially available for malt wort fermentation industries and present results confirm its suitability for such purposes. Nevertheless, measures should be adopted to exploit its response to added Mg^{2+} in malt wort fermentation.

Experimental evidence from fermentations of different types of Mg^{2+}-supplemented molasses further confirmed the inducible effects of the cation on fermentation. Although all of the fermented molasses were rich in total Mg^{2+}, ethanol production was stimulated by adding Mg^{2+}. Added Mg^{2+} (100ppm) into 30% w/v solutions of Danish beet, Brazilian, Javan, Mexican, Pakistani, South African and Sri Lankan sugarcane molasses fermentations increased ethanol production by 1.37, 0.91, 0.76, 0.68, 0.72, 0.67, 0.46, respectively over the controls. The above availability studies confirm that although the studied industrial and synthetic media were rich in total Mg^{2+}, sufficient Mg^{2+} is not available for yeast fermentative metabolism. This could be due either to the interactions between other ions, chelation, adhesion to the cumulative effect of all those phenomena.

Mg^{2+} uptake studies revealed that yeast cells have a very high demand for this cation during the early hours of growth and fermentation. However, further studies revealed that the major part of Mg^{2+} taken up during exponential phase was effluxed from the cells during stationary phase of growth. These results lead to the conclusion that for active growth and fermentation yeast cells require more Mg^{2+} than the resting cells. The above findings support Dombek and Ingram (1986) who pointed out that the decline of fermentation towards the end of batch fermentations was caused by Mg^{2+} deficiency and Mg^{2+} supplementation prolonged the exponentional phase of growth, increased biomass and cell viability. Further, Mg^{2+}-supplemented cultutres achieved higher cellular Mg^{2+} levels at the

CHAPTER 7: REFERENCES

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Appendix 1

Analytical parameters for Mg^{2+} and Ca^{2+} determination by AAS

Programme - Mg and Ca,

Elements - Mg and Ca,

Wave length - 285.2

Technique - AA-BG

Signal processing - Hold

Reading delay - sec. 0.0

Replicates - 3

Integration time - 1 sec.

Printer - data

Oxidant - air

Oxidant flow - 8 (l/min)

Fuel flow -2.5 (l/min)

Lamp current - MA 12

Appendix 2

Analytical parameters for Zn^{2+} determination by AAS

Programme -Zn,

Element - Zn

Wave length - 213.2

Technique - AA-BG

Signal processing - Hold

Reading delay - sec. 0.0

Replicates - 3

Integration time - 1 sec.

Printer - data

Oxidant - air

Oxidant flow - 8 (l/min)

Fuel flow -2.5 (l/min)

Lamp current - MA 15

The three published papers cited below have been removed from the e-thesis due to copyright restrictions:

Walker, G.M., Birch, R.M., Chandrasena, G. and Manyard, A.I. (1996). Magnesium, Calcium, and Fermentative Metabolism in Industrial Yeasts. In: *Journal of American Society of Brewing Chemists,* 54(1), pp.13-18.

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